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## Box Patent Application

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Presented for filing is a new original patent application of:

**Applicant:** JACK R. WANDS, SUZANNE M. DE LA MONTE,  
NEDIM INCE and ROLF I. CARLSON

**Title:** DIAGNOSIS AND TREATMENT OF MALIGNANT  
NEOPLASMS

Enclosed are the following papers, including those required to receive a filing date under 37 CFR §1.53(b):

	<u>Pages</u>
Specification	52
Claims	5
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Signed Declaration	[To Be Filed At A Later Date]
Drawings	9

Enclosures:

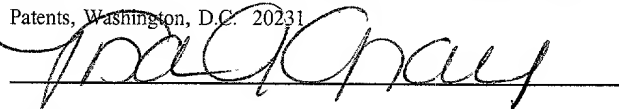
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Page 2

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Enclosures

APPLICATION  
FOR  
UNITED STATES LETTERS PATENT

04930-032001

TITLE: DIAGNOSIS AND TREATMENT OF MALIGNANT  
NEOPLASMS

APPLICANT: JACK R. WANDS, SUZANNE M. DE LA MONTE,  
NEDIM INCE and ROLF I. CARLSON

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DIAGNOSIS AND TREATMENT OF MALIGNANT NEOPLASMS

Statement as to Federally Sponsored Research

5           This invention was made with U.S. Government support under National Institutes of Health grants CA-35711, AA-02666, AA-02169, and AA11431. The government has certain rights in the invention.

Background of the Invention

10           Primary malignant central nervous system (CNS) neoplasms, particularly glioblastomas, are highly fatal due to their aggressive and widespread infiltration of the brain and resistance to anti-cancer treatments. Although progress has been made in unraveling the pathological mechanisms  
15           underlying CNS cancers as well as other cancer types, tumor specific therapeutic approaches and methods of diagnosis have been largely elusive.

Summary of the Invention

20           The invention features a method for diagnosing a malignant neoplasm in a mammal by contacting a bodily fluid from the mammal with an antibody which binds to an human aspartyl (asparaginy) beta-hydroxylase (HAAH) polypeptide under conditions sufficient to form an antigen-antibody complex and detecting the antigen-antibody complex.  
25           Malignant neoplasms detected in this manner include those derived from endodermal tissue, e.g., colon cancer, breast cancer, pancreatic cancer, liver cancer, and cancer of the bile ducts. Neoplasms of the central nervous system (CNS) such as primary malignant CNS neoplasms of both neuronal and  
30           glial cell origin and metastatic CNS neoplasms are also detected. Patient derived tissue samples, e.g., biopsies of solid tumors, as well as bodily fluids such as a CNS-derived bodily fluid, blood, serum, urine, saliva, sputum, lung effusion, and ascites fluid, are contacted with an HAAH-  
35           specific antibody.

04930-1030

The assay format is also useful to generate temporal data used for prognosis of malignant disease. A method for prognosis of a malignant neoplasm of a mammal is carried out by (a) contacting a bodily fluid from the mammal with an antibody which binds to an HAAH polypeptide under conditions sufficient to form an antigen-antibody complex and detecting the antigen-antibody complex; (b) quantitating the amount of complex to determine the level of HAAH in the fluid; and (c) comparing the level of HAAH in the fluid with a normal control level of HAAH. An increasing level of HAAH over time indicates a progressive worsening of the disease, and therefore, an adverse prognosis.

The invention also includes an antibody which binds to HAAH. The antibody preferably binds to a site in the carboxyterminal catalytic domain of HAAH. Alternatively, the antibody binds to an epitope that is exposed on the surface of the cell. The antibody is a polyclonal antiserum or monoclonal antibody. The invention encompasses not only an intact monoclonal antibody, but also an immunologically-active antibody fragment, e. g. , a Fab or (Fab)<sub>2</sub> fragment; an engineered single chain Fv molecule; or a chimeric molecule, e.g., an antibody which contains the binding specificity of one antibody, e.g., of murine origin, and the remaining portions of another antibody, e.g., of human origin. Preferably the antibody is a monoclonal antibody such as FB50, 5C7, 5E9, 19B, 48A, 74A, 78A, 86A, HA238A, HA221, HA 239, HA241, HA329, or HA355. Antibodies which bind to the same epitopes as those monoclonal antibodies are also within the invention.

An HAAH-specific intrabody is a recombinant single chain HAAH-specific antibody that is expressed inside a target cell, e.g., tumor cell. Such an intrabody binds to endogenous intracellular HAAH and inhibits HAAH enzymatic

activity or prevents HAAH from binding to an intracellular ligand. HAAH-specific intrabodies inhibit intracellular signal transduction, and as a result, inhibit growth of tumors which overexpress HAAH.

5           A kit for diagnosis of a tumor in a mammal contains an HAAH-specific antibody. The diagnostic assay kit is preferentially formulated in a standard two-antibody binding format in which one HAAH-specific antibody captures HAAH in a patient sample and another HAAH-specific antibody is used  
10 to detect captured HAAH. For example, the capture antibody is immobilized on a solid phase, e.g., an assay plate, an assay well, a nitrocellulose membrane, a bead, a dipstick, or a component of an elution column. The second antibody, i.e., the detection antibody, is typically tagged with a  
15 detectable label such as a colorimetric agent or radioisotope.

          Also within the invention is a method of inhibiting tumor growth in a mammal, which is carried out by administering to the mammal a compound which inhibits  
20 expression or enzymatic activity of HAAH. Preferably, the compound is substantially pure nucleic acid molecule such as an HAAH antisense DNA, the sequence of which is complementary to a coding sequence of HAAH. Expression of HAAH is inhibited by contacting mammalian cells, e.g., tumor  
25 cells, with HAAH antisense DNA or RNA, e.g., a synthetic HAAH antisense oligonucleotide. For example, HAAH antisense nucleic acid is introduced into glioblastoma cells or other tumor cells which overexpress HAAH. Binding of the antisense nucleic acid to an HAAH transcript in the target  
30 cell results in a reduction in HAAH production by the cell. By the term "antisense nucleic acid" is meant a nucleic acid (RNA or DNA) which is complementary to a portion of an mRNA, and which hybridizes to and prevents translation of the

mRNA. Preferably, the antisense DNA is complementary to the 5' regulatory sequence or the 5' portion of the coding sequence of HAAH mRNA (e.g., a sequence encoding a signal peptide or a sequence within exon 1 of the HAAH gene).

5 Standard techniques of introducing antisense DNA into the cell may be used, including those in which antisense DNA is a template from which an antisense RNA is transcribed. The method is to treat tumors in which expression of HAAH is upregulated, e.g., as a result of malignant transformation  
10 of the cells. The length of the oligonucleotide is at least 10 nucleotides and may be as long as the naturally-occurring HAAH transcript. Preferably, the length is between 10 and 50 nucleotides, inclusive. More preferably, the length is between 10 and 20 nucleotides, inclusive.

15 By "substantially pure DNA or RNA" is meant that the nucleic acid is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank a HAAH gene. The term therefore includes, for example, a recombinant nucleic acid which is  
20 incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a procaryote or eucaryote at a site other than its natural site; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease  
25 digestion) independent of other sequences. It also includes a recombinant nucleic acid which is part of a hybrid gene encoding additional polypeptide sequence such as a nucleic acid encoding an chimeric polypeptide, e.g., one encoding an antibody fragment linked to a cytotoxic polypeptide.

30 Alternatively, HAAH expression is inhibited by administering a ribozyme or a compound which inhibits binding of Fos or Jun to an HAAH promoter sequence.

Compounds, which inhibit an enzymatic activity of HAAH, are useful to inhibit tumor growth in a mammal. By enzymatic activity of HAAH is meant hydroxylation of an epidermal growth factor (EGF)-like domain of a polypeptide.

5 For example an EGF-like domain has the consensus sequence CX<sub>7</sub>CX<sub>4</sub>CX<sub>10</sub>CXCX<sub>8</sub>C (SEQ ID NO:1). HAAH hydroxylase activity is inhibited intracellularly. For example, a dominant negative mutant of HAAH (or a nucleic acid encoding such a mutant) is administered. The dominant negative HAAH mutant contains a

10 mutation which changes a ferrous iron binding site from histidine of a naturally-occurring HAAH sequence to a non-iron-binding amino acid, thereby abolishing the hydroxylase activity of HAAH. The histidine to be mutated, e.g., deleted or substituted, is located in the carboxyterminal

15 catalytic domain of HAAH. For example, the mutation is located between amino acids 650-700 (such as the His motif, underlined sequence of SEQ ID NO:2) the native HAAH sequence. For example, the mutation is at residues 671, 675, 679, or 690 of SEQ ID NO:2. An HAAH-specific intrabody

20 is also useful to bind to HAAH and inhibit intracellular HAAH enzymatic activity, e.g., by binding to an epitope in the catalytic domain of HAAH. Other compounds such as L-mimosine or hydroxypyridone are administered directly into a tumor site or systemically to inhibit HAAH hydroxylase

25 activity.

Table 1: Amino acid sequence of HAAH

	MAQRKNAKSS	GNSSSSGSGS	GSTSAGSSSP	GARRETKHGG	HKNGRKGGLS	GTSFFTWFMV	61
	IALLGWTSV	AVVWFDLVDY	EEVLGKLGIV	DADGDGDFDV	DDAKVLLGLK	ERSTSEPAVP	121
	PEEAEPHTEP	EEQVPVEAEP	QNIIDEAKEQ	IQSLLHEMVH	AEHVEGEDLQ	QEDGPTGEPQ	181
30	QEDDEFLMAT	DVDDRFETLE	PEVSHEETEH	SYHVEETVSQ	DCNQDMEEMM	SEQENPDSSE	241
	PVVEDERLHH	DTDDVTYQVY	EEQAVYEPL	NEGIEITEVT	APPEDNPVED	SQVIVEEVSI	301
	FPVEEQQEV	PETNRKTTDD	EQKAKVKKKK	PKLLNKFDKT	IKAELDAAEK	LRKRKIEEA	361
	VNAFKELVRK	YPQSPRARYG	KAQCEDDLAE	KRRSNEVLRG	AIETYQEVAS	LPDVPADLLK	421
	LSLKRRSDRQ	QFLGHMRGSL	LTLQRLVQLF	PNDTSLKNDL	GVGYLLIGDN	DNAKKVYEEV	481
35	LSVTPNDGFA	KVHYGFILKA	QNKIAESIPY	LKEGIESGDP	GTDDGRFYFH	LGDAMQRVGN	541
	KEAYKWYELG	HKRGHFASVW	QRLSYNVNGL	KAQPWWTPKE	TGYTELVKSL	ERNWKLIRDE	601
	GLAVMDKAKG	LFLPEDENLR	EKGDSQFTL	WQQGRRNENA	CKGAPKTCTL	LEKFPETTC	661
	RRGQIKYSIM	HPGTHVWPHT	GPTNCRLRMH	LGLVIPKEGC	KIRCANETRT	WEEGKVLIFD	721

DSFEHEVWQD ASSFRLIFIV DVWHPELTPQ QRRSLPAI (SEQ ID NO:2; GENBANK  
Accession No. S83325; His motif is underlined; conserved  
sequences within the catalytic domain are designated by bold  
type)

5 For example, a compound which inhibits HAAH  
hydroxylation is a polypeptide that binds a HAAH ligand but  
does not transduce an intracellular signal or an polypeptide  
which contains a mutation in the catalytic site of HAAH.  
Such a polypeptide contains an amino acid sequence that is  
10 at least 50% identical to a naturally-occurring HAAH amino  
acid sequence or a fragment thereof and which has the  
ability to inhibit HAAH hydroxylation of substrates  
containing an EGF-like repeat sequence. More preferably,  
the polypeptide contains an amino acid sequence that is at  
15 least 75%, more preferably at least 85%, more preferably at  
least 95% identical to SEQ ID NO: .

A substantially pure HAAH polypeptide or HAAH-  
derived polypeptide such as a mutated HAAH polypeptide is  
preferably obtained by expression of a recombinant nucleic  
20 acid encoding the polypeptide or by chemically synthesizing  
the protein. A polypeptide or protein is substantially pure  
when it is separated from those contaminants which accompany  
it in its natural state (proteins and other naturally-  
occurring organic molecules). Typically, the polypeptide is  
25 substantially pure when it constitutes at least 60%, by  
weight, of the protein in the preparation. Preferably, the  
protein in the preparation is at least 75%, more preferably  
at least 90%, and most preferably at least 99%, by weight,  
HAAH. Purity is measured by any appropriate method, e.g.,  
30 column chromatography, polyacrylamide gel electrophoresis,  
or HPLC analysis. Accordingly, substantially pure  
polypeptides include recombinant polypeptides derived from a  
eucaryote but produced in *E. coli* or another procaryote, or

in a eucaryote other than that from which the polypeptide was originally derived.

Nucleic acid molecules which encode such HAAH or HAAH-derived polypeptides are also within the invention.

Table 2: HAAH cDNA sequence

(SEQ ID NO:3 ; GENBANK Accession No. S83325; codon encoding initiating methionine is underlined).

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5      cggaccgtgc aatggcccag cgtaagaatg ccaagagcag cggcaacagc agcagcagcg 61
      gctccggcag cggtagcacg agtgcgggca gcagcagccc cggggcccgg agagagacaa 121
      agcatggagg acacaagaat gggaggaaag gcggaactctc gggaacttca ttcttcacgt 181
10     ggtttatggt gattgcattg ctgggcgtct ggacatctgt agctgtcgtt tggtttgatc 241
      ttgttgacta tgaggaagtt ctaggaaaac taggaatcta tgatgctgat ggtgatggag 301
      attttgatgt ggatgatgcc aaagttttat taggacttaa agagagatct acttcagagc 361
      cagcagtccc gccagaagag gctgagccac acactgagcc cgaggagcag gttcctgtgg 421
      aggcagaacc ccagaatatc gaagatgaag caaaagaaca aattcagtc cttctccatg 481
      aaatggtaca cgcagaacat gttgagggag aagacttgca acaagaagat ggaccacacag 541
15     gagaaccaca acaagaggat gatgagtttc ttatggcgac tgatgtagat gatagatttg 601
      agaccctgga acctgaagta tctcatgaag aaaccgagca tagttaccac gtggaagaga 661
      cagtttcaca agactgtaat caggatatgg aagagatgat gtctgagcag gaaaatccag 721
      attccagtga accagtagta gaagatgaaa gattgcacca tgatacagat gatgtaacat 781
      accaagtcta tgaggaacaa gcagtatatg aacctctaga aaatgaaggg atagaaatca 841
20     cagaagtaac tgctccccct gaggataatc ctgtagaaga ttcacaggta attgtagaag 901
      aagtaagcat ttttctgtg gaagaacagc aggaagtacc accagaaaca aatagaaaaa 961
      cagatgatcc agaacaaaaa gcaaaagtta agaaaaagaa gcctaaactt ttaaataaat 1021
      ttgataagac tattaagct gaacttgatg ctgcagaaaa actccgtaaa aggggaaaaa 1081
      ttgaggaagc agtgaatgca tttaaagaac tagtacgcaa ataccctcag agtccacgag 1141
25     caagatatgg gaaggcgcag tgtgaggatg atttggctga gaagaggaga agtaatgagg 1201
      tgctacgtgg agccatcgag acctaccaag aggtggccag cctacctgat gtccctgcag 1261
      acctgctgaa gctgagtttg aagcgtcgct cagacaggca acaatttcta ggtcatatga 1321
      gaggttccct gcttaccctg cagagattag ttcaactatt tccaatgat acttccttaa 1381
      aaaatgacct tggcgtggga tacctcttga taggagataa tgacaatgca aagaaagttt 1441
30     atgaagaggt gctgagtgtg acacctaatg atggctttgc taaagtccat tatggcttca 1501
      tcctgaaggc acagaacaaa attgctgaga gcattcccata tttaaaggaa ggaatagaat 1561
      cgggagatcc tggcactgat gatgggagat tttatttcca cctgggggat gccatgcaga 1621
      ggggttgggaa caaagaggca tataagtggg atgagcttgg gcacaagaga ggacactttg 1681
      catctgtctg gcaacgctca ctctacaatg tgaatggact gaaagcacag ccttgggtgga 1741
35     ccccaaaaga aacgggctac acagagttag taaagtcttt agaaagaaac tggaaagttaa 1801
      tccgagatga aggccttgca gtgatggata aagccaaagg tctcttctg cctgaggatg 1861
      aaaacctgag ggaaaaaggg gactggagcc agttcacgct gtggcagcaa ggaagaagaa 1921
      atgaaaatgc ctgcaaagga gctcctaaaa cctgtacctt actagaaaag ttccccgaga 1981
      caacaggatg cagaagagga cagatcaaat attccatcat gcaccccggg actcacgtgt 2041
40     ggccgcacac agggcccaca aactgcaggc tccgaatgca cctgggcttg gtgattccca 2101
      aggaaggctg caagattcga tgtgccaacg agaccaggac ctggggaggaa ggcaaggtgc 2161
      tcattcttga tgactcctt gagcacgagg tatggcagga tgctcatct ttccggctga 2221
      tattcatcgt ggatgtgtgg catccggaac tgacaccaca gcagagacgc agccttccag 2281
      caatttagca tgaattcatg caagcttggg aaactctgga gaga
45     (SEQ ID NO:3 ; GENBANK Accession No. S83325; codon encoding
      initiating methionine is underlined).

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Methods of inhibiting tumor growth also include administering a compound which inhibits HAAH hydroxylation of a NOTCH polypeptide. For example, the compound inhibits hydroxylation of an EGF-like cysteine-rich repeat sequence in a NOTCH polypeptide, e.g., one containing the consensus

sequence CDXXXCXXXGNGXCDXXCNNAACXXDGXDC (SEQ ID NO:4).

Polypeptides containing an EGF-like cysteine-rich repeat sequence are administered to block hydroxylation of endogenous NOTCH.

5 Growth of a tumor which overexpresses HAAH is also inhibited by administering a compound which inhibits signal transduction through the insulin receptor substrate (IRS) signal transduction pathway. Preferably the compound inhibits IRS phosphorylation. For example, the compound is  
10 a peptide or non-peptide compound which binds to and inhibits phosphorylation at residues 46, 465, 551, 612, 632, 662, 732, 941, 989, or 1012 of SEQ ID NO:5. Compounds include polypeptides such those which block an IRS phosphorylation site such as a Glu/Tyr site. Antibodies  
15 such as those which bind to a carboxyterminal domain of IRS containing a phosphorylation site block IRS phosphorylation, and as a consequence, signal transduction along the pathway. Inhibition of IRS phosphorylation in turn leads to inhibition of cell proliferation. Other compounds which  
20 inhibit IRS phosphorylation include vitamin D analogue EB1089 and Wortmannin.

HAAH-overproducing tumor cells were shown to express HAAH both intracellularly and on the surface of the tumor cell. Accordingly, a method of killing a tumor cell is  
25 carried out by contacting such a tumor cell with a cytotoxic agent linked to an HAAH-specific antibody. The HAAH-specific antibody (antibody fragment, or ligand which binds to extracellular HAAH) directs the chimeric polypeptide to the surface of the tumor cell allowing the cytotoxic agent  
30 to damage or kill the tumor cell to which the antibody is bound. The monoclonal antibody binds to an epitope of HAAH such as an epitope exposed on the surface of the cell or in

the catalytic site of HAAH. The cytotoxic composition preferentially kills tumor cells compared to non-tumor cell.

Screening methods to identify anti-tumor agents which inhibit the growth of tumors which overexpress HAAH are also within the invention. A screening method used to determine whether a candidate compound inhibits HAAH enzymatic activity includes the following steps: (a) providing a HAAH polypeptide, e.g., a polypeptide which contains the carboxyterminal catalytic site of HAAH; (b) providing a polypeptide comprising an EGF-like domain; (c) contacting the HAAH polypeptide or the EGF-like polypeptide with the candidate compound; and (d) determining hydroxylation of the EGF-like polypeptide of step (b). A decrease in hydroxylation in the presence of the candidate compound compared to that in the absence of said compound indicates that the compound inhibits HAAH hydroxylation of EGF-like domains in proteins such as NOTCH.

Anti-tumor agents which inhibit HAAH activation of NOTCH are identified by (a) providing a cell expressing HAAH; (b) contacting the cell with a candidate compound; and (c) measuring translocation of activated NOTCH to the nucleus of said cell. Translocation is measured by using a reagent such as an antibody which binds to a 110 kDa activation fragment of NOTCH. A decrease in translocation in the presence of the candidate compound compared to that in the absence of the compound indicates that the compound inhibits HAAH activation of NOTCH, thereby inhibiting NOTCH-mediated signal transduction and proliferation of HAAH-overexpressing tumor cells.

Nucleotide and amino acid comparisons described herein were carried out using the Lasergene software package (DNASTAR, Inc., Madison, WI). The MegAlign module used was the Clustal V method (Higgins et al., 1989, CABIOS 5(2):151-

153). The parameter used were gap penalty 10, gap length penalty 10.

Hybridization is carried out using standard techniques, such as those described in Ausubel et al.

(*Current Protocols in Molecular Biology*, John Wiley & Sons, 1989). "High stringency" refers to nucleic acid hybridization and wash conditions characterized by high temperature and low salt concentration, e.g., wash conditions of 65°C at a salt concentration of 0.1 X SSC.

"Low" to "moderate" stringency refers to DNA hybridization and wash conditions characterized by low temperature and high salt concentration, e.g., wash conditions of less than 60°C at a salt concentration of at least 1.0 X SSC. For example, high stringency conditions include hybridization at 42°C in the presence of 50% formamide; a first wash at 65°C in the presence of 2 X SSC and 1% SDS; followed by a second wash at 65°C in the presence of 0.1% x SSC. Lower stringency conditions suitable for detecting DNA sequences having about 50% sequence identity to an HAAH gene sequence are detected by, for example, hybridization at about 42°C in the absence of formamide; a first wash at 42°C, 6 X SSC, and 1% SDS; and a second wash at 50°C, 6 X SSC, and 1% SDS.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Brief Description of the Drawings

Fig. 1 is a bar graph showing colony formation induced by transient transfection of NIH-3T3 cells with various AAH cDNAs. Colony formation was induced by transient transfection with 10 µg DNA. In contrast, the mutant murine AAH construct without enzymatic activity has no transforming activity. The data is presented as mean number of transformed foci ± SEM.

Fig. 2 is a bar graph showing the results of a densitometric analysis of a Western blot assay of proteins produced by various murine AAH stably transfected cell clones. In clones 7 and 18, there was a modest increase in HAAH gene expression, while the overexpression was to a lesser degree in clone 16.

Figs. 3A-B are bar graphs showing colony formation in soft agar exhibited by HAAH stably transfected clones compared to HAAH enzymatic activity. Fig. 3A shows a measurement of murine AAH enzymatic activity in clones 7, 16 and 18, and Fig. 3B shows colony formation exhibited by clones 7, 16 and 18. Data is presented as mean number of colonies 10 days after plating  $\pm$  SEM. All three clones with modest increases in HAAH enzymatic activity, that correlated with protein expression, exhibited anchorage independent growth.

Fig. 4 is a bar graph showing tumor formation in nude mice injected with transfected clones overexpressing murine AAH. Tumor growth was assessed after 30 days. Mean tumor weight observed in mice injected with clones 7, 16 and 18 as compared to mock DNA transfected clone. All animals injected with clones overexpressing HAAH developed tumors.

Figs. 5A-D are bar graphs showing increased AAH expression in PNET2 (Fig. 5A, 5C) and SH-Sy5y (Fig. 5B) cells treated with retinoic acid (Figs. 5A, 5B) or phorbol ester myristate (PMA; Fig. 5C) to induce neurite outgrowth as occurs during tumor cell invasion. The cells were treated with 10  $\mu$ M retinoic acid or 100 nM PMA for 0, 1, 2, 3, 4, or 7 days. Cell lysates were analyzed by Western blot analysis using an HAAH-specific monoclonal antibody to detect the 85 kDa AAH protein. The levels of immunoreactivity were measured by volume densitometry (arbitrary units). The graphs indicate the mean  $\pm$  S.D. of

results obtained from three separate experiments. In Fig. 5D, PNET2 cells were treated for 24 hours with sub-lethal concentrations of H<sub>2</sub>O<sub>2</sub> to induce neurite retraction. Viability of greater than 90% of the cells was demonstrated by Trypan blue dye exclusion. Similar results were obtained for SH-Sy5y cells.

Fig. 6 is a bar graph showing the effects of AAH over-expression on the levels of anti-apoptosis (Bcl-2), cell cycle-mitotic inhibitor (p16 and p21/Waf1), and proliferation (proliferating cell nuclear antigen; PCNA) molecules. PNET2 neuronal cells were stably transfected with the full-length human cDNA encoding AAH (pHAAH) or empty vector (pcDNA). AAH gene expression was under control of a CMV promoter. Western blot analysis was performed with cell lysates prepared from cultures that were 70 to 80 percent confluent. Protein loading was equivalent in each lane. Replicate blots were probed with the different antibodies. Bar graphs depict the mean S.D.'s of protein expression levels measured in three experiments. All differences are statistically significant by Student T-test analysis (P<0.01-P<0.001).

Fig. 7 is a diagram of showing the components of the IRS-1 signal transduction pathway.

Fig. 8 is a line graph showing growth curves generated in cells expressing the antisense HAAH compared to controls expressing GFP.

Fig. 9 is a diagram of the functional domains of the hIRS-1 protein and structural organization of the point mutants. All mutant and "wild type" hIRS-1 proteins construct contain a FLAG (F) epitope (DYKDDDDK; SEQ ID NO:7) at the C-terminus. PH and PTB indicate pleckstrin homology and phosphotyrosine binding, regions, respectively.

#### Detailed Description

HAAH is a protein belonging to the ( $\alpha$ -ketoglutarate dependent dioxygenase family of prolyl and lysyl hydroxylases which play a key role in collagen biosynthesis. This molecule hydroxylates aspartic acid or asparagine

5 residues in EGF-like domains of several proteins in the presence of ferrous iron. These EGF-like domains contain conserved motifs, that form repetitive sequences in proteins such as clotting factors, extracellular matrix proteins, LDL receptor, NOTCH homologues or NOTCH ligand homologues.

10 The alpha-ketoglutarate-dependent dioxygenase aspartyl (asparaginyl) beta-hydroxylase (AAH) specifically hydroxylates one aspartic or asparagine residue in EGF-like domains of various proteins. The 4.3-kb cDNA encoding the human AspH (hAspH) hybridizes with 2.6 kb and 4.3 kb

15 transcripts in transformed cells, and the deduced amino acid sequence of the larger transcript encodes an protein of about 85 kDa. Both *in vitro* transcription and translation and Western blot analysis also demonstrate a 56-kDa protein that may result from posttranslational cleavage of the

20 catalytic C terminus.

An physiological function of AAH is the post-translational beta-hydroxylation of aspartic acid in vitamin K-dependent coagulation proteins. However, the abundant expression of AAH in several malignant neoplasms, and low levels of AAH in many normal cells indicate a role

25 for this enzyme in malignancy. The AAH gene is also highly expressed in cytotrophoblasts, but not syncytiotrophoblasts of the placenta. Cytotrophoblasts are invasive cells that mediate placental implantation. The increased levels of AAH

30 expression in human cholangiocarcinomas, hepatocellular carcinomas, colon cancers, and breast carcinomas were primarily associated with invasive or metastatic lesions. Moreover, overexpression of AAH does not strictly reflect

increased DNA synthesis and cellular proliferation since high levels of AAH immunoreactivity were observed in 100 percent of cholangiocarcinomas, but not in human or experimental disease processes associated with regeneration or nonneoplastic proliferation of bile ducts. AAH overexpression and attendant high levels of beta hydroxylase activity lead to invasive growth of transformed neoplastic cells. Detection of an increase in HAAH expression is useful for early and reliable diagnosis of the cancer types which have now been characterized as overexpressing this gene product.

#### Diagnosis of malignant tumors

HAAH is overexpressed in many tumors of endodermal origin and in at least 95% of CNS tumors compared to normal noncancerous cells. An increase in HAAH gene product in a patient-derived tissue sample (e.g., solid tissue or bodily fluid) is carried out using standard methods, e.g., by Western blot assays or a quantitative assay such as ELISA. For example, a standard competitive ELISA format using an HAAH-specific antibody is used to quantify patient HAAH levels. Alternatively, a sandwich ELISA using a first antibody as the capture antibody and a second HAAH-specific antibody as a detection antibody is used.

Methods of detecting HAAH include contacting a component of a bodily fluid with an HAAH-specific antibody bound to solid matrix, e.g., microtiter plate, bead, dipstick. For example, the solid matrix is dipped into a patient-derived sample of a bodily fluid, washed, and the solid matrix is contacted with a reagent to detect the presence of immune complexes present on the solid matrix.

Proteins in a test sample are immobilized on (bound to) a solid matrix. Methods and means for covalently or noncovalently binding proteins to solid matrices are known

in the art. The nature of the solid surface may vary depending upon the assay format. For assays carried out in microtiter wells, the solid surface is the wall of the well or cup. For assays using beads, the solid surface is the surface of the bead. In assays using a dipstick (i.e., a solid body made from a porous or fibrous material such as fabric or paper) the surface is the surface of the material from which the dipstick is made. Examples of useful solid supports include nitrocellulose (e.g., in membrane or microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates, polyvinylidene fluoride (known as IMMULON™), diazotized paper, nylon membranes, activated beads, and Protein A beads. The solid support containing the antibody is typically washed after contacting it with the test sample, and prior to detection of bound immune complexes. Incubation of the antibody with the test sample is followed by detection of immune complexes by a detectable label. For example, the label is enzymatic, fluorescent, chemiluminescent, radioactive, or a dye. Assays which amplify the signals from the immune complex are also known in the art, e.g., assays which utilize biotin and avidin.

An HAAH-detection reagent, e.g., an antibody, is packaged in the form of a kit, which contains one or more HAAH-specific antibodies, control formulations (positive and/or negative), and/or a detectable label. The assay may be in the form of a standard two-antibody sandwich assay format known in the art.

#### Production of HAAH-specific antibodies

Anti-HAAH antibodies were obtained by techniques well known in the art. Such antibodies are polyclonal or monoclonal. Polyclonal antibodies were obtained, for example, by the methods described in Ghose et al., Methods

in Enzymology, Vol. 93, 326-327, 1983. An HAAH polypeptide, or an antigenic fragment thereof, was used as the immunogen to stimulate the production of polyclonal antibodies in the antisera of rabbits, goats, sheep, or rodents. Antigenic polypeptides for production of both polyclonal and monoclonal antibodies useful as immunogens include polypeptides which contain an HAAH catalytic domain. For example, the immunogenic polypeptide is the full-length mature HAAH protein or an HAAH fragment containing the carboxyterminal catalytic domain e.g., an HAAH polypeptide containing the His motif of SEQ ID NO:2.

Antibodies which bind to the same epitopes as those antibodies disclosed herein as identified using standard methods, e.g., competitive binding assays, known in the art.

Monoclonal antibodies were obtained by standard techniques. Ten  $\mu$ g of purified recombinant HAAH polypeptide was administered to mice intraperitoneally in complete Freund's adjuvant, followed by a single boost intravenously (into the tail vein) 3-5 months after the initial inoculation. Antibody-producing hybridomas were made using standard methods. To identify those hybridomas producing antibodies that are highly specific for an HAAH polypeptide, hybridomas were screened using the same polypeptide immunogen used to immunize. Those antibodies which were identified as having HAAH-binding activity are also screened for the ability to inhibit HAAH catalytic activity using the enzymatic assays described below. Preferably, the antibody has a binding affinity of at least about  $10^8$  liters/mole and more preferably, an affinity of at least about  $10^9$  liters/mole.

Monoclonal antibodies are humanized by methods known in the art, e.g, MAbs with a desired binding specificity can

be commercially humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA).

HAAH-specific intrabodies are produced as follows. Following identification of a hybridoma producing a suitable monoclonal antibody, DNA encoding the antibody is cloned. DNA encoding a single chain HAAH-specific antibody in which heavy and light chain variable domains are separated by a flexible linker peptide is cloned into an expression vector using known methods (e.g., Marasco et al., 1993, Proc. Natl. Acad. Sci. USA 90:7889-7893 and Marasco et al., 1997, Gene Therapy 4:11-15). Such constructs are introduced into cells, e.g., using standard gene delivery techniques for intracellular production of the antibodies.

Intracellular antibodies, i.e., intrabodies, are used to inhibit signal transduction by HAAH. Intrabodies which bind to a carboxyterminal catalytic domain of HAAH inhibit the ability of HAAH to hydroxylate EGF-like target sequences.

Methods of linking HAAH-specific antibodies (or fragments thereof) which bind to cell surface exposed epitopes of HAAH on the surface of a tumor cell are linked to known cytotoxic agents, e.g, ricin or diptheria toxin, using known methods.

#### Methods of treating malignant tumors

Patients with tumors characterized as overexpressing HAAH as such tumors of endodermal origin or CNS tumors are treated by administering HAAH antisense nucleic acids.

Antisense therapy is used to inhibit expression of HAAH in patients suffering from hepatocellular carcinomas, cholangiocarcinomas, glioblastomas and neuroblastomas. For example, an HAAH antisense strand (either RNA or DNA) is directly introduced into the cells in a form that is capable of binding to the mRNA transcripts. Alternatively, a vector containing a sequence which, which once within the target

cells, is transcribed into the appropriate antisense mRNA, may be administered. Antisense nucleic acids which hybridize to target mRNA decrease or inhibit production of the polypeptide product encoded by a gene by associating with the normally single-stranded mRNA transcript, thereby interfering with translation and thus, expression of the protein. For example, DNA containing a promoter, e.g., a tissue-specific or tumor specific promoter, is operably linked to a DNA sequence (an antisense template), which is transcribed into an antisense RNA. By "operably linked" is meant that a coding sequence and a regulatory sequence(s) (i.e., a promoter) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

Oligonucleotides complementary to various portions of HAAH mRNA are tested *in vitro* for their ability to decrease production of HAAH in tumor cells (e.g., using the FOCUS hepatocellular carcinoma (HCC) cell line) according to standard methods. A reduction in HAAH gene product in cells contacted with the candidate antisense composition compared to cells cultured in the absence of the candidate composition is detected using HAAH-specific antibodies or other detection strategies. Sequences which decrease production of HAAH in *in vitro* cell-based or cell-free assays are then be tested *in vivo* in rats or mice to confirm decreased HAAH production in animals with malignant neoplasms.

Antisense therapy is carried out by administering to a patient an antisense nucleic acid by standard vectors and/or gene delivery systems. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes

viruses, retroviruses, adenoviruses and adeno-associated viruses, among others. A reduction in HAAH production results in a decrease in signal transduction via the IRS signal transduction pathway. A therapeutic nucleic acid composition is formulated in a pharmaceutically acceptable carrier. The therapeutic composition may also include a gene delivery system as described above. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal: e.g., physiological saline. A therapeutically effective amount of a compound is an amount which is capable of producing a medically desirable result such as reduced production of an HAAH gene product or a reduction in tumor growth in a treated animal.

Parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal delivery routes, may be used to deliver nucleic acids or HAAH-inhibitory peptides or non-peptide compounds. For treatment of CNS tumors, direct infusion into cerebrospinal fluid is useful. The blood-brain barrier may be compromised in cancer patients, allowing systemically administered drugs to pass through the barrier into the CNS. Liposome formulations of therapeutic compounds may also facilitate passage across the blood-brain barrier.

Dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular nucleic acid to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosage for intravenous administration of nucleic acids is from approximately  $10^6$  to  $10^{22}$  copies of the nucleic acid molecule.

Ribozyme therapy is also be used to inhibit HAAH gene expression in cancer patients. Ribozymes bind to specific mRNA and then cut it at a predetermined cleavage point, thereby destroying the transcript. These RNA molecules are used to inhibit expression of the HAAH gene according to methods known in the art (Sullivan et al., 1994, J. Invest. Derm. 103:85S-89S; Czubayko et al., 1994, J. Biol. Chem. 269:21358-21363; Mahieu et al, 1994, Blood 84:3758-65; Kobayashi et al. 1994, Cancer Res. 54:1271-1275).

Methods of identifying compounds that inhibit HAAH enzymatic activity

Aspartyl (asparaginyl) beta-hydroxylase (AAH) activity is measured *in vitro* or *in vivo*. For example, HAAH catalyzes posttranslational modification of  $\beta$  carbon of aspartyl and asparaginyl residues of EGF-like polypeptide domains. An assay to identify compounds which inhibit hydroxylase activity is carried out by comparing the level of hydroxylation in an enzymatic reaction in which the candidate compound is present compared to a parallel reaction in the absence of the compound (or a predetermined control value). Standard hydroxylase assays carried out in a testtube are known in the art, e.g., Lavaissiere et al., 1996, J. Clin. Invest. 98:1313-1323; Jia et al., 1992, J. Biol. Chem. 267:14322-14327; Wang et al., 1991, J. Biol. Chem. 266:14004-14010; or Gronke et al., 1990, J. Biol. Chem. 265:8558-8565. Hydroxylase activity is also measured using carbon dioxide ( $^{14}\text{CO}_2$  capture assay) in a 96-well microtiter plate format (Zhang et al., 1999, Anal. Biochem. 271:137-142. These assays are readily automated and suitable for high throughput screening of candidate compounds to identify those with hydroxylase inhibitory activity.

Candidate compound which inhibit HAAH activation of NOTCH are identified by detecting a reduction in activated NOTCH in a cell which expresses or overexpresses HAAH, e.g., FOCUS HCC cells. The cells are cultured in the presence of a candidate compound. Parallel cultures are incubated in the absence of the candidate compound. To evaluate whether the compound inhibits HAAH activation of NOTCH, translocation of activated NOTCH to the nucleus of the cell is measured. Translocation is measured by detecting a 110 kDa activation fragment of NOTCH in the nucleus of the cell. The activation fragment is cleaved from the large (approximately 300 kDa) transmembrane NOTCH protein upon activation. Methods of measuring NOTCH translocation are known, e.g, those described by Song et al., 1999, Proc. Natl. Acad. Sci U.S.A. 96:6959-6963 or Capobianco et al., 1997, Mol. Cell Biol. 17:6265-6273. A decrease in translocation in the presence of the candidate compound compared to that in the absence of the compound indicates that the compound inhibits HAAH activation of NOTCH, thereby inhibiting NOTCH-mediated signal transduction and proliferation of HAAH-overexpressing tumor cells.

Methods of screening for compounds which inhibit phosphorylation of IRS are carried out by incubating IRS-expressing cells in the presence and absence of a candidate compound and evaluating the level of IRS phosphorylation in the cells. A decrease in phosphorylation in cells cultured in the presence of the compound compared to in the absence of the compound indicates that the compound inhibits IRS-1 phosphorylation, and as a result, growth of HAAH-overexpressing tumors. Alternatively, such compounds are identified in an *in vitro* phosphorylation assay known in the art, e.g., one which measured phosphorylation of a synthetic substrate such as poly (Glu/Tyr).

Example 1: Increased expression of HAAH is associated with malignant transformation

HAAH is a highly conserved enzyme that hydroxylates EGF-like domains in transformation associated proteins. The HAAH gene is overexpressed in human hepatocellular carcinomas and cholangiocarcinomas. HAAH gene expression was found to be undetectable during bile duct proliferation in both human disease and rat models compared to cholangiocarcinoma. Overexpression of HAAH in NIH-3T3 cells was associated with generation of a malignant phenotype, and enzymatic activity was found to be required for cellular transformation. The data described below indicate that overexpression of HAAH is linked to cellular transformation of biliary epithelial cells.

To identify molecules that are specifically overexpressed in transformed malignant cells of human hepatocyte origin, the FOCUS hepatocellular carcinoma (HCC) cell line was used as an immunogen to generate monoclonal antibodies (mAb) that specifically or preferentially recognize proteins associated with the malignant phenotype. A lambda GT11 cDNA expression library derived from HepG2 HCC cells was screened, and HAAH-specific mAb produced against the FOCUS cell line was found to recognize an epitope on a protein encoded by an HAAH cDNA. The HAAH enzyme was found to be upregulated in several different human transformed cell lines and tumor tissues compared to adjacent human tissue counterparts. The overexpressed HAAH enzyme in different human malignant tissues was found to be catalytically active.

HAAH gene expression was examined in proliferating bile ducts and in NIH 3T3 cells. Its role in the generation of the malignant phenotype was measured by the formation of transformed foci, growth in soft agar as an index of

anchorage independent growth and tumor formation in nude mice. The role of enzymatic activity in the induction of transformed phenotype was measured by using a cDNA construct with a mutation in the catalytic site that abolished  
5 hydroxylase activity. The results indicated that an increase in expression of HAAH gene is associated with malignant transformation of bile ducts.

The following materials and methods were used to generate the data described below.

#### Antibodies

The FB50 monoclonal antibody was generated by cellular immunization of Balb/C mice with FOCUS HCC cells. A monoclonal anti-Dengue virus antibody was used as a non-relevant control. The HBOH2 monoclonal antibody was  
15 generated against a 52 kDa recombinant HAAH polypeptide and recognizes the catalytic domain of beta-hydroxylase from mouse and human proteins. Polyclonal anti-HAAH antibodies cross-react with rat hydroxylase protein. Control antibody anti-Erk-1 was purchased from Santa Cruz Biotechnology, Inc,  
20 CA. Sheep anti-mouse and donkey anti-rabbit antisera labeled with horseradish peroxidase were obtained from Amersham, Arlington Heights, IL.

#### Constructs

The murine full length AAH construct (pNH376) and  
25 the site-directed mutation construct (pNH376-H660) with abolished catalytic activity were cloned into the eukaryotic expression vector pcDNA3 (Invitrogen Corp., San Diego, CA). The full length human AAH was cloned into prokaryotic expression vector pBC-SK+ (Stratagene, La Jolla, CA). The  
30 full length human AAH (GENBANK Accession No. S83325) was subcloned into the EcoRI site of the pcDNA3 vector.

#### Animal model of bile duct proliferation



Liver tissue sections (5  $\mu$ m) were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was quenched by a 30-minute treatment with 0.6 % H<sub>2</sub>O<sub>2</sub> in 60% methanol. Endogenous biotin was masked by incubation with avidin-biotin blocking solutions (Vector Laboratories, Burlingame, CA). The FB50 mAb (for PSC samples) and polyclonal anti-HAAH-hydroxylase antibodies (for rat liver samples) were added to slides in a humidified chamber at 4°C overnight. Immunohistochemical staining was performed using a standard avidin-biotin horseradish peroxidase complex (ABC) method using Vectastain Kits with diaminobenzidine (DAB) as the chromogen according to manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA). Tissue sections were counterstained with hematoxylin, followed by dehydration in ethanol. Sections were examined by a light microscopy for bile duct proliferation and HAAH protein expression. Paraffin sections of cholangiocarcinoma and placenta were used as positive controls, and hepatosteatosi samples were used as a negative controls. To control for antibody binding specificity, adjacent sections were immunostained in the absence of a primary antibody, or using non-relevant antibody to Dengue virus. As a positive control for tissue immunoreactivity, adjacent sections of all specimens were immunostained with monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase.

#### Western blot analysis

Cell lysates were prepared in a standard radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. The total amount of protein in the lysates was determined by Bio-Rad colorimetric assay (Bio Rad, Hercules, CA) followed by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE),

transferred to PVDF membranes, and subjected to Western blot analysis using FB50, HBOH2, anti-Erk-1 (used as an internal control for protein loading) as primary, sheep anti-mouse and donkey anti-rabbit antisera labeled with horseradish peroxidase as secondary antibodies. Antibody binding was detected with enhanced chemiluminescence reagents (SuperSignal, Pierce Chemical Company, Rockford, IL) and film autoradiography. The levels of immunoreactivity were measured by volume densitometry using NIH Image software.

#### Enzymatic activity assay

AAH activity was measured in cell lysates using the first EGF-like domain of bovine protein S as substrate where  $^{14}\text{C}$ -labeled  $\alpha$ -ketoglutarate hydroxylates the domain releasing  $^{14}\text{C}$  containing  $\text{CO}_2$  according to standard methods, e.g., those described by Jia et al., 1992, J. Biol. Chem. 267:14322-14327; Wang et al., 1991, J. Biol. Chem. 266:14004-14010; or Gronke et al., 1990, J. Biol. Chem. 265:8558-8565. Incubations were carried out at  $37^\circ\text{C}$  for 30 min in a final volume of  $40\ \mu\text{l}$  containing  $48\ \mu\text{g}$  of crude cell extract protein and  $75\ \mu\text{M}$  EGF substrate.

#### Cell transfection studies

The NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Washington, DC) supplemented with 10 % heat-inactivated fetal calf serum (FCS; Sigma Chemical Co., St. Louis, MO), 1% L-glutamine, 1% non-essential amino acids and 1% penicillin-streptomycin (GIBCO BRL, Life Technologies, Inc., Grand Island, NY). Subconfluent NIH-3T3 cells ( $3 \times 10^5$  cells/60-mm dish) were transfected with  $10\ \mu\text{g}$  of one of the following plasmids:

- 1) non-recombinant pcDNA3 vector (Invitrogen Corp., San Diego, CA) as a negative control; 2) pNH376-H660, the murine AAH cDNA that was mutated in the catalytic domain and cloned into the pcDNA3 vector driven by a CMV promoter; 3) pNH376,

the wild type murine AAH cDNA cloned into the pCDNA3 vector;  
4) pCDHH, wild type human AAH cDNA cloned into the pCDNA3  
vector; or 5) pLNCX-UP1, a cDNA that encodes v-Src oncogene  
(positive control). Cells were transfected using the

5 calcium phosphate transfection kit according to  
manufacturer's instructions (5 Prime - 3 Prime, Inc.,  
Boulder, CO). Comparison of cellular transfection  
efficiency was assessed with the various constructs. For  
this procedure, confluent plates obtained 48 hours after  
10 transfection were split and reseeded into 12 separate 6-cm  
dishes, and 6 of them were made to grow in the presence of  
400  $\mu$ g/ml G-418 (GIBCO BRL, Life Technologies, Inc., Grant  
Island, NY) containing medium. The number of G-418  
resistant foci was determined at 14 days after transfection  
15 and used to correct for any variability in transfection  
efficiency.

#### Transformation assay

The NIH-3T3 cells were transfected with the various  
constructs and allowed to reach confluence after 48 hours as  
20 described above. Each 6 cm dish was split and seeded into  
12 different 6 cm dishes. While 6 of them were made to grow  
in the presence of G-418 to detect transfection efficiency,  
the other six were grown in complete medium without G-418  
and with a medium change every 4th day. The number of  
25 transformed foci were counted in these plates without G-418  
and expressed as transformed foci per  $\mu$ g transfected DNA.

#### Anchorage-independent cell growth assay

A limiting dilution technique (0.15 cell/well of a  
flat bottom 96-well-plate) was performed on transfectants  
30 grown in G-418 in order to isolate cell clones with  
different levels of HAAH activity as measured by Western  
blot analysis and enzymatic assay of hydroxylase activity.  
Cloned cell lines ( $1.0 \times 10^4$  cells) were suspended in

complete medium containing 0.4 % low-melting agarose  
(SeaPlaque GTG Agarose; FMC Bioproducts, Rockland, Maine)  
and laid over a bottom agar mixture consisting of complete  
medium with 0.53 % low-melting agarose. Each clone was  
5 assayed in triplicate. The clones were seeded under these  
conditions and 10 days later the size (positive growth > 0.1  
mm in diameter) and number of foci were determined.

#### Tumorigenicity in nude mice

The same clones as assessed in the anchorage  
10 independent growth assay were injected into nude mice and  
observed for tumor formation. Tumorigenicity was evaluated  
using 10 animals in each of 4 groups (Charles River Labs.,  
Wilmington, MA). Group 1 received  $1 \times 10^7$  cells stably  
transfected with mock DNA, Group 2-4 received  $1 \times 10^7$  cells  
15 of clones stable transfected with pNH376 and expressing  
various levels of murine HAAH protein. Nude mice were kept  
under pathogen-free conditions in a standard animal  
facility. Thirty days after tumor cell inoculation, the  
animals were sacrificed using isofluorane (Aerrane,  
20 Anaquest, NJ) containing chambers and the tumors were  
carefully removed and weight determined.

#### Animal model of bile duct proliferation

Following ligation of the common bile duct,  
intrahepatic bile duct proliferation was evident at 48  
25 hours. Tissue samples obtained 8 and 16 days following  
common bile duct ligation revealed extensive bile duct  
proliferation as shown in Table 3.

Table 3: Bile duct proliferation and HAAH expression  
at different intervals after common bile duct ligation

Group	Surgical Procedure	Microscopy*	Immunohisto- chemistry
1	no surgery	normal	negative

2	sham surgery	normal	negative
3	6 hours post ligation	normal	negative
4	12 hours post ligation	normal	negative
5	24 hours post ligation	normal	negative
6	48 hours post ligation	minimal bile duct prolif.	negative
7	4 days post ligation	moderate bile duct prolif.	negative
8	8 days post ligation	extensive bile duct prolif.	negative
9	16 days post ligation	extensive bile duct prolif.	negative

\* Investigation was performed under light microscopy following a hematoxylin and eosin staining.

Immunohistochemical staining failed to detect presence of HAAH in proliferating bile ducts at any time. Analysis of HAAH expression in bile ducts derived from sham surgical controls was also negative, while all samples exhibited positive immunoreactivity with control antibodies to glyceraldehyde 3-phosphate dehydrogenase. Thus, bile duct proliferation was not associated with increased HAAH expression in this standard animal model system.

#### HAAH expression in PSC

The liver biopsy specimens from patients with PSC exhibited bile duct proliferation accompanied by periductal fibrosis and a mononuclear inflammatory cell infiltrate without evidence of dysplasia. Adjacent sections immunostained with the an HAAH-specific monoclonal antibody had no detectable HAAH immunoreactivity in proliferating

bile ducts. In contrast, sections of cholangiocarcinoma that were immunostained simultaneously using the same antibody and detection reagents manifested intense levels of HAAH immunoreactivity in nearly all tumor cells, whereas adjacent sections of the cholangiocarcinomas exhibited a negative immunostaining reaction with monoclonal antibody to Dengue virus. These findings indicate that HAAH expression was associated with malignant transformation rather than non-cancerous cellular proliferation of intrahepatic bile ducts.

#### HAAH associated transformation of NIH-3T3 cells

The transforming capability of the murine and human AAH genes, as well as the murine AAH mutant construct without enzymatic activity were compared to mock DNA (negative control) and v-Src transfected NIH-3T3 cells (positive control). The transforming capability of murine AAH was found to be 2-3 times that of vector DNA control as shown in Fig. 1. The transforming capacity of the human gene was greater than that observed with the murine AAH ( $32 \pm 1.5$  versus  $13 \pm 2.6$  transformed foci, respectively). The murine and human AAH transfected cells formed large foci, resembling those of v-Src transfected fibroblasts, compared to the occasional much smaller foci observed in cells transfected with vector DNA that displayed the contact inhibition of fibroblast cell lines. Parallel experiments performed using the mutant pNH376-H660 construct without enzymatic activity revealed no transforming activity. This finding indicates that the enzymatic activity of HAAH is required for the transforming activity exhibited by the HAAH gene.

#### Anchorage-independent cell growth assay

After transient transfection with the murine AAH construct, several different transformed foci were isolated

for dilutional cloning experiments to establish stable transfected cell clones with different levels of HAAH gene expression. Nine different cloned cell lines were selected for further study. The expression level of the HAAH protein was determined by Western blot analysis. Clones 7 and 18 had a modest increase in HAAH protein expression, yet formed large colonies in soft agar (Fig. 2). Protein loading was equivalent in all lanes as shown by immunoblotting of the same membranes with an anti-Erk-1 monoclonal antibody. The increased protein expression was associated with increased enzymatic activity as shown in Fig. 3. The capability of these clones to exhibit anchorage independent cell growth in soft agar is presented in Fig. 3. All 3 clones with increased HAAH gene expression demonstrated anchorage independent cell growth compared to the mock DNA transfected clone.

#### Tumor formation in nude mice

The 3 clones with increased HAAH gene expression were evaluated for the ability to form tumors in nude mice. Tumor size in the mouse given clone 18 was compared to a mock DNA transfected clone. Clones 7, 16 and 18 were highly transformed in this assay and produced large tumors with a mean weight of 2.5, 0.9 and 1.5 grams, respectively (Fig. 4). These data indicate that overexpression of HAAH contributes to induction and maintenance of the malignant phenotype *in vivo*.

#### High level HAAH expression is indicative of malignancy

In order to determine if HAAH expression was associated with malignancy rather than increased cell turnover, two models of bile duct proliferation were studied. In the animal model, ligation of the common bile duct induced extensive intrahepatic bile duct proliferation,

yet there was no evidence of HAAH gene expression under these experimental conditions as shown in Table 3.

Similarly, HAAH gene expression was assessed in a human disease model associated with bile duct proliferation since  
5   PSC is an autoimmune liver disease associated with destruction as well as proliferation of the intra and extrahepatic bile ducts. PSC is premalignant disease, and a significant proportion of affected individuals will eventually develop cholangiocarcinoma. However, no evidence  
10   for increased HAAH gene expression in the presence of extensive bile duct proliferation.

Having established that HAAH protein levels were elevated in cholangiocarcinoma and not in normal or proliferating bile ducts, the role of HAAH in the generation  
15   of a malignant phenotype was studied. The HAAH gene was transfected into NIH-3T3 cells and cellular changes, e.g., increased formation of transformed foci, colony growth in soft agar and tumor formation in nude mice associated with malignant transformation, were evaluated. The full-length  
20   murine and human AAH genes were cloned into expression constructs and transiently transfected into NIH-3T3 cells. An increased number of transformed foci was detected in cells transfected both with the murine and human AAH genes as compared to mock DNA transfected controls. The increased  
25   number of transformed foci, after controlling for transfection efficiency, was not as high compared to v-Src gene transfected cells used as a positive control. The enzymatic activity of the HAAH gene was required for a malignant phenotype because a mutant construct which  
30   abolished the catalytic site had no transforming properties. Several stable transfectants and cloned NIH-3T3 cell lines with a modest increase in HAAH protein levels and enzymatic activity were established. Such cell lines were placed in

soft agar to examine anchorage independent cell growth as another property of the malignant phenotype. All cell lines grew in soft agar compared to mock DNA transfected control, and there was a positive correlation between the cellular  
5 level of HAAH gene expression and the number and size of colonies formed. Three of these cloned cell lines formed tumors in nude mice. All three cell lines with increased HAAH expression were oncogenic as shown by the development of large tumors as another well-known characteristic of the  
10 transformed phenotype.

To determine whether cellular changes induced by overexpression of HAAH were related to the enzymatic function, a site-directed mutation was introduced into the gene that changed the ferrous iron binding site from  
15 histidine to lysine at 660th position of mouse HAAH thereby abolishing hydroxylase activity of the murine HAAH. A corresponding mutation in HAAH is used as a dominant negative mutant to inhibit HAAH hydroxylase activity. The pNH376-H660 construct had no transformation activity  
20 indicating cellular changes of the malignant phenotype induced by overexpression depends on the enzymatic activity of the protein.

Notch receptors and their ligands have several EGF-like domains in the N-terminal region that contain the  
25 putative consensus sequence for beta-hydroxylation. Notch ligands are important elements of the Notch signal transduction pathway and interaction of Notch with its ligands occurs by means of EGF-like domains of both molecules. Point mutations affecting aspartic acid or  
30 asparagine residues in EGF-like domains that are the targets for beta-hydroxylation by HAAH reduce calcium binding and protein-protein interactions involved in the activation of downstream signal transduction pathways. Overexpression of

HAAH and Notch protein hydroxylation by HAAH contributes to malignancy. Tumor growth is inhibited by decreasing Notch protein hydroxylation by HAAH

The data presented herein is evidence that high-level HAAH expression is linked to malignant transformation. An increase in expression of the HAAH cDNA in NIH-3T3 cells induced a transformed phenotype manifested by increased numbers of transformed foci, anchorage-independent growth, and tumorigenesis in nude mice. In addition, intact HAAH-enzyme was found to be required for HAAH-associated transformation. Accordingly, inhibition of as little as 20% of endogenous HAAH enzymatic activity or expression confers a therapeutic benefit. For example, clinical benefit is achieved by 50%-70% inhibition of HAAH expression or activity after administration of an HAAH inhibitory compound compared to the level associated with untreated cancer cell or a normal noncancerous cell.

HAAH is regulated at the level of transcription. Only modest increases in HAAH expression and enzyme activity were required for cellular transformation. These results indicate that increased HAAH gene expression and enzyme activity contribute to the generation or maintenance of the transformed phenotype and that decreasing transcription of the HAAH gene or decreasing enzymatic activity of the HAAH gene product leads to a decrease in malignancy. Accordingly, HAAH transcription is inhibited by administering compounds which decrease binding of Fos and/or Jun (elements which regulate HAAH transcription) to HAAH promoter sequences.

Since HAAH is up-regulated with malignant transformation of bile duct epithelium, and HAAH immunoreactivity is detectable on tumor cell surface membranes, HAAH is also a molecule to which to target a

cytotoxic agent, e.g., by linking the cytotoxic agent to a compound that binds to HAAH expressed on the surface of a tumor cell. Assay of HAAH protein levels in either biological fluids such as bile, or cells obtained by fine needle aspiration is a diagnostic marker of human cholangiocarcinoma.

Example 2: Expression of AAH and growth and invasiveness of malignant CNS neoplasms

AAH is abundantly expressed in carcinomas and trophoblastic cells, but not in most normal cells, including those of CNS origin. High levels of AAH expression were observed in 15 of 16 glioblastomas, 8 of 9 anaplastic oligodendrogliomas, and 12 of 12 primitive neuroectodermal tumors (PNETs). High levels of AAH immunoreactivity were primarily localized at the infiltrating edges rather than in the central portions of tumors. Double-label immunohistochemical staining demonstrated a reciprocal relationship between AAH and tenascin, a substrate for AAH enzyme activity. PNET2 neuronal cell lines treated with phorbol ester myristate or retinoic acid to stimulate neuritic extension and invasive growth exhibited high levels of AAH expression, whereas H<sub>2</sub>O<sub>2</sub>-induced neurite retraction resulted in down-regulation of AAH. PNET2 neuronal cells that stably over-expressed the human AAH cDNA had increased levels of PCNA and Bcl-2, and reduced levels of p21/Waf1 and p16, suggesting that AAH overexpression results in enhanced pathological cell proliferation, cell cycle progression, and resistance to apoptosis. In addition, the reduced levels of p16 observed in AAH-transfectants indicate that AAH over-expression confers enhanced invasive growth of neoplastic cells since deletion or down-regulation of the p16 gene correlates with more aggressive and invasive in vivo growth of glioblastomas. Increased AAH

immunoreactivity was detected at the infiltrating margins of primary malignant CNS neoplasms, further indicating a role of HAAH in tumor invasiveness.

The following materials and methods were used to generate the data described below.

Analysis of AAH Immunoreactivity in Primary Human Malignant CNS Neoplasms:

AAH immunoreactivity was examined in surgical resection specimens of glioblastoma (N=16), anaplastic oligodendroglioma (N=9), and primitive neuroectodermal tumor (PNET; supratentorial neuroblastomas (N=3) and medulloblastomas (N=9). The histopathological sections were reviewed to confirm the diagnoses using standard criteria. Paraffin sections from blocks that contained representative samples of viable solid tumor, or tumor with adjacent intact tissue were studied. Sections from normal adult postmortem brains (N=4) were included as negative controls. AAH immunoreactivity was detected using qn HAAH-specific monoclonal antibody. Immunoreactivity was revealed by the avidin-biotin horseradish peroxidase complex method (Vector ABC Elite Kit; Vector Laboratories, Burlingame, CA) using 3-3' diaminobenzidine (DAB) as the chromogen (24) and hematoxylin as a counterstain.

Tenascin and laminin are likely substrates for AAH due to the presence of EGF-like repeats within the molecules. Double-immunostaining studies were performed to co-localize AAH with tenascin or laminin. The AAH immunoreactivity was detected by the ABC method with DAB as the chromogen, and tenascin or laminin immunoreactivity was detected by the avidin-biotin alkaline phosphatase complex method (Vector Laboratories, Burlingame, CA) with BCIP/NBT as the substrate. As positive and negative controls, adjacent sections were immunostained with monoclonal

antibody to glial fibrillary acidic protein (GFAP) and Hepatitis B surface antigen. All specimens were batch immunostained using the same antibody dilutions and immunodetection reagents.

#### 5            Cell Lines and Culture Conditions

Studies were conducted to determine whether AAH expression was modulated with neurite (filopodia) extension (sprouting) as occurs with invasive growth of malignant neoplasms. Human PNET2 CNS-derived and SH-Sy5y neuroblastoma cells were cultured and stimulated for 0, 1, 10 2, 3, 5, or 7 days with 100 nM phorbol 12-ester 13-acetate or 10  $\mu$ M retinoic acid to induce sprouting. In addition, to examine the effects of neurite retraction on AAH expression, subconfluent cultures were treated for 24 hours with low 15 concentrations (10-40  $\mu$ M) of H<sub>2</sub>O<sub>2</sub>. For both studies, AAH expression was evaluated by Western blot analysis using the an HAAH-specific antibody.

#### Generation of PNET2 AAH-transfected Clones

The full-length human AAH cDNA (SEQ ID NO:3) was 20 ligated into the pcDNA3.1 mammalian expression vector in which gene expression was under the control of a CMV promoter (Invitrogen Corp., San Diego, CA). PNET2 cells were transfected with either pHAAH or pcDNA3 (negative control) using Cellfectin reagent (Gibco BRL, Grand Island, 25 NY). Neomycin-resistant clones were selected for study if the constitutive levels of AAH protein expression were increased by at least two-fold relative to control (pcDNA3) as detected by Western blot analysis. To determine how AAH overexpression altered the expression of genes that modulate 30 the transformed phenotype, the levels of proliferating cell nuclear antigen (PCNA), p53, p21/Waf1, Bcl-2, and p16 were measured in cell lysates prepared from subconfluent cultures of AAH (N=5) and pcDNA3 (N=5) stably transfected clones.

PCNA was used as marker of cell proliferation. p53, p21/Waf1, and Bcl-2 levels were examined to determine whether cells that over-expressed AAH were more prone to cell cycle progression and more resistant to apoptosis. The levels of p16 were assessed to determine whether AAH over-expression has a role in tumor invasiveness.

#### Western blot analysis

Cells grown in 10 cm<sup>2</sup> dishes were lysed and homogenized in a standard radioimmunoprecipitation assay RIPA buffer containing protease and phosphatase inhibitors. The supernatants collected after centrifuging the samples at 12,000 x g for 10 minutes to remove insoluble debris were used for Western blot analysis. Protein concentration was measured using the BCA assay (Pierce Chemical Co, Rockford, IL). Samples containing 60 µg of protein were electrophoresed in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and subjected to Western blot analysis. Replicate blots were probed with the individual antibodies. Immunoreactivity was detected with horseradish peroxidase conjugated IgG (Pierce Chemical Co, Rockford, IL) and enhanced chemiluminescence reagents. To quantify the levels of protein expression, non-saturated autoradiographs were subjected to volume densitometry using NIH Image software, version 1.6. Statistical comparisons between pHAH and pcDNA3 transfected cells were made using Student T tests.

#### Antibodies

HAAH-specific monoclonal antibody generated against the FOCUS hepatocellular carcinoma cells were used to detect AAH immunoreactivity. Monoclonal antibodies to tenascin, and glial fibrillary acidic protein, and rabbit polyclonal antibody to laminin were purchased from Sigma Co (St. Louis, MO). Rabbit polyclonal antibody to human p16 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The

5C3 negative control monoclonal antibody to Hepatitis B surface antigen was generated using recombinant protein and used as a negative control.

#### AAH immunoreactivity in primary malignant brains

##### 5 tumors

AAH immunoreactivity was detected in 15 of 16 glioblastomas, 8 of 9 anaplastic oligodendrogliomas, and all 12 PNETs. AAH immunoreactivity was localized in the cytoplasm, nucleus, and cell processes. The tissue  
10 distribution of AAH immunoreactivity was notable for the intense labeling localized at the interfaces between tumor and intact brain, and the conspicuously lower levels of immunoreactivity within the central portions of the tumors. High levels of AAH immunoreactivity were also observed in  
15 neoplastic cells distributed in the subpial zones, leptomeninges, Virchow-Robin perivascular spaces, and in individual or small clusters of neoplastic cells that infiltrated the parenchyma. In contrast, AAH immunoreactivity was not detectable in normal brain. The  
20 distribution of AAH immunoreactivity appeared not to be strictly correlated with DNA synthesis since the density of nuclei in mitosis (1-5%) was similar in the central and peripheral portions of the tumors.

#### Relationship between AAH and tenascin

##### 25 immunoreactivity in glioblastomas

Tenascin is an extracellular matrix-associated antigen expressed in malignant gliomas. Tenascin contains EGF-like domains within the molecule, a substrate for HAAH hydroxylation. To localize AAH in relation to tenascin  
30 immunoreactivity in malignant brain tumors, double-label immunohistochemical staining was performed in which AAH was detected using a brown chromogen (DAB), and tenascin, a blue chromogen (BCIP/NBT). Adjacent sections were similarly

double-labeled to co-localize AAH with laminin, another EGF domain containing extracellular matrix molecule expressed in the CNS. Intense levels of tenascin immunoreactivity were observed in perivascular connective tissue and in association with glomeruloid proliferation of endothelial cells. The double-labeling studies demonstrated a reciprocal relationship between AAH and tenascin immunoreactivity such that high levels of AAH were associated with low or undetectable tenascin, and low levels of AAH were associated with abundant tenascin immunoreactivity. Although laminins are also likely substrates for AAH enzyme activity due to the EGF repeats within the molecules, double labeling studies revealed only low levels of laminin immunoreactivity throughout the tumors and at interfaces between tumor and intact tissue.

Analysis of AAH expression in neuronal cell lines treated with PMA or RA

Neuritic sprouting/filopodia extension marks invasive growth of neoplastic neuronal cells. PMA activates protein kinase C signal transduction pathways that are involved in neuritic sprouting. Retinoic acid binds to its own receptor and the ligand-receptor complex translocates to the nucleus where it binds to specific consensus sequences present in the promoter/enhancer regions of target genes involved in neuritic growth. Both PNET2 and SH-Sy5y cells can be induced to sprout by treatment with PMA (60-120 nM) or retinoic acid (5-10  $\mu$ M). Figs. 5A-D depict data from representative Western blot autoradiographs; the bar graphs correspond to the mean  $\pm$  S.D. of results obtained from three experiments. Western blot analysis with the FB50 antibody detected doublet bands corresponding to protein with an molecular mass of approximately 85 kDa. Untreated PNET2 cells had relatively low levels of AAH immunoreactivity

(Fig. 5A), whereas untreated SH-Sy5y cells had readily detected AAH expression (Fig. 5B). Untreated PNET2 cells exhibited polygonal morphology with coarse, short radial cell processes, whereas SH-Sy5y cells were slightly elongated and spontaneously extend fine tapered processes. Both cell lines manifested time-dependent increases in the levels of AAH immunoreactivity following either RA (Figs. 5A and 5B) or PMA (Fig. 5C) stimulation and neurite extension. In PNET2 cells, the levels of AAH protein increased by at least two-fold 24 hours after exposure to RA or PMA, and high levels of AAH were sustained throughout the 7 days of study. In SH-Sy5y cells, the RA- or PMA-stimulated increases in AAH expression occurred more gradually and were highest after 7 days of treatment (Fig. 5B).

To examine the effect of AAH expression on neurite retraction, PNET2 and SH-Sy5y cells were treated with low concentrations (8-40  $\mu$ M) of  $H_2O_2$ . After 24 hours exposure to up to 40  $\mu$ M  $H_2O_2$ , although most cells remained viable (Trypan blue dye exclusion), they exhibited neurite retraction and rounding. Western blot analysis using the FB50 antibody demonstrated  $H_2O_2$  dose-dependent reductions in the levels of AAH protein (Fig. 5D).

#### Effects of AAH over-expression in PNET2 cells

To directly assess the role of AAH overexpression in relation to the malignant phenotype, PNET2 cells were stably transfected with the human full-length cDNA with gene expression under control of a CMV promoter (pHAAH). Neomycin-resistant clones that had at least two-fold higher levels of AAH immunoreactivity relative to neomycin-resistant pcDNA3 (mock) clones were studied. Since aggressive behavior of malignant neoplasms is associated with increased DNA synthesis, cell cycle progression, resistance to apoptosis, and invasive growth, the changes in

phenotype associated with constitutive over-expression of AAH were characterized in relation to PCNA, p21/Waf1, p53, Bcl-2, and p16. PCNA was used as an index of DNA synthesis and cell proliferation. p21/Waf1 is a cell cycle inhibitor.

5 Expression of the p53 tumor-suppressor gene increases prior to apoptosis, whereas bcl-2 inhibits apoptosis and enhances survival of neuronal cells. p16 is an oncosuppressor gene that is often either down-regulated or mutated in infiltrating malignant neoplasms.

10 Five pHAAH and 5 pcDNA3 clones were studied. Increased levels of AAH expression in the pHAAH transfected clones was confirmed by Western (Fig. 6) and Northern blot analyses. Western blot analysis using cell lysates from cultures that were 70 to 80 percent confluent demonstrated

15 that constitutively increased levels of AAH expression (approximately 85 kDa;  $P < 0.05$ ) in pHAAH-transfected cells were associated with significantly increased levels of PCNA (approximately 35 kDa;  $P < 0.01$ ) and Bcl-2 (approximately 25 kDa;  $P < 0.05$ ), and reduced levels of p21/Waf1

20 (approximately 21 kDa;  $P < 0.001$ ) and p16 (approximately 16 kDa;  $P < 0.001$ ) (Fig. 6). However, the pHAAH stable transfectants also exhibited higher levels of wild-type p53 (approximately 53-55 kDa). Although AAH expression (85 kDa protein) in the stable transfectants was increased by only

25 75 to 100 percent, the levels of p16 and p21/Waf1 were sharply reduced, and PCNA increased by nearly two-fold (Fig. 6).

Increased AAH expression is indicative of growth and invasiveness of malignant CNS neoplasms

30 The data described herein demonstrates that AAH overexpression is a diagnostic tool by which to identify primary malignant CNS neoplasms of both neuronal and glial cell origin. Immunohistochemical staining studies

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demonstrated that AAH overexpression was detectable mainly at the interfaces between solid tumor and normal tissue, and in infiltrating neoplastic cells distributed in the subpial zones, leptomeninges, perivascular spaces, and parenchyma.

5 *In vitro* experiments demonstrated that AAH gene expression was modulated with neurite (filopodium) extension and invasiveness and down-regulated with neurite retraction. In addition, PNET2 cells stably transfected with the AAH cDNA exhibited increased PCNA and bcl-2, and reduced Waf1/p21 and  
10 p16 expression. Therefore, AAH overexpression contributes to the transformed phenotype of CNS cells by modulating the expression of other genes that promote cellular proliferation and cell cycle progression, inhibit apoptosis, or enhance tumor cell invasiveness.

15 The data demonstrated readily detectable AAH mRNA transcripts (4.3 kB and 2.6 kB) and proteins (85 kDa and 50-56 kDa) in PNET2 and SH-Sy5y cells, but not in normal brain. Correspondingly, high levels of AAH immunoreactivity were observed in 35 of the 37 in malignant primary  
20 CNS-derived neoplasms studied, whereas the 4 normal control brains had no detectable AAH immunoreactivity. The presence of high-level AAH immunoreactivity at the infiltrating margins and generally not in the central portions of the tumors indicates that AAH overexpression is involved in the  
25 invasive growth of CNS neoplasms. Administration of compounds which decrease AAH expression or enzymatic activity inhibits proliferation of CNS tumors which overexpress AAH, as well as metastases of CNS tumors to other tissue types.

30 The AAH enzyme hydroxylates EGF domains of a number of proteins. Tenascin, an extracellular matrix molecule that is abundantly expressed in malignant gliomas, contains EGF-like domains. Since tenascin promotes tumor cell

invasion, its abundant expression in glioblastomas represents an autocrine mechanism of enhanced tumor cell growth vis-à-vis the frequent overexpression of EGF or EGF-like receptors in malignant glial cell neoplasms.

- 5 Analysis of the functional domains of tenascins indicated that the mitogenic effects of this family of molecules are largely mediated by the fibronectin domains, and that the EGF-like domains inhibit growth, cell process elongation, and matrix invasion. Therefore, hydroxylation of the
- 10 EGF-like domains by AAH represents an important regulatory factor in tumor cell invasiveness.

Double-label immunohistochemical staining studies demonstrated a reciprocal relationship between AAH and tenascin immunoreactivity such that high levels AAH

15 immunoreactivity present at the margins of tumors were associated with low levels of tenascin, and low levels of AAH were often associated with high levels of tenascin. These observations indicated that AAH hydroxylation of EGF-like domains of tenascin alters the immunoreactivity of

20 tenascin protein, and in so doing, facilitates the invasive growth of malignant CNS neoplasms into adjacent normal tissue and perivascular spaces.

AAH immunoreactivity was examined in PNET2 and SH-Sy5y neuronal cells induced to undergo neurite extension

25 with PMA or retinoic acid, or neurite retraction by exposure to low doses of  $H_2O_2$ . AAH expression was sharply increased by PMA- or retinoic acid-induced neurite (filopodium) extension, and inhibited by  $H_2O_2$ -induced neurite retraction and cell rounding. Neurite or filopodium extension and

30 attachment to extracellular matrix are required for tumor cell invasion in the CNS. The EGF-like domains of tenascin inhibit neuritic and glial cell growth into the matrix during development.

To directly examine the role of AAH overexpression in relation to the transformed phenotype, genes modulated with DNA synthesis, cell cycle progression, apoptosis, and tumor invasiveness were examined in neuronal cell clones that stably over-expressed the human AAH cDNA. The findings of increased PCNA and reduced Waf1/p21 immunoreactivity indicated that AAH overexpression enhances cellular proliferation and cell cycle progression. In addition, the finding of increased Bcl-2 expression indicated that AAH overexpression contributes to the transformed phenotype by increasing cellular resistance to apoptosis. The apparently contradictory finding of higher levels of p53 in the cells that overexpressed AAH is explained by the observation that high levels of wildtype p53 in immature neuronal cells were associated with neuritic growth (invasiveness) rather than apoptosis. Levels of p16 were reduced (compared to normal cells) or virtually undetectable in cells that constitutively overexpressed AAH; a deletion mutation of the p16 gene has been correlated with invasive growth and more rapid progression of malignant neoplasms, including those of CNS origin. These data indicate that p16 expression is modulated by AAH.

Example 3: Increased HAAH production and IRS-mediated signal transduction

IRS-1 mediated signal transduction pathway is activated in 95% of human HCC tumors compared to the adjacent uninvolved liver tissue. HAAH is a downstream effector gene involved in this signal transduction pathway. HAAH gene upregulation is closely associated with overexpression of IRS-1 in HCC tumors as revealed by immunohistochemical staining and Western blot analysis. A high level of HAAH protein is expressed in HCC and cholangiocarcinoma compared to normal hepatocytes and bile

ducts. Both of these tumors also exhibit high level expression of IRS-1 by immunohistochemical staining. FOCUS HCC cell clones stably transfected with a C-terminal truncated dominant negative mutant of IRS-1, which blocks insulin and IGF-1 stimulated signal transduction, was associated with a striking reduction in HAAH gene expression in liver. In contrast, transgenic mice overexpressing IRS-1 demonstrate an increase in HAAH gene expression by Western blot analysis. Insulin stimulation of FOCUS HCC cells (20 and 40 U) in serum free medium and after 16 hr of serum starvation demonstrated upregulation of HAAH gene expression. These data indicate that HAAH gene expression is a downstream effector of the IRS-1 signal transduction pathway.

Example 4: Effects of HAAH expression levels on the characteristics of the malignant phenotype

Overexpression of IRS-1 in NIH 3T3 cells induces transformation. The full-length murine HAAH construct was cloned into the pcDNA3 eukaryotic expression vector. A second murine construct encoded HAAH with abolished catalytic activity due to a site directed mutation. The full-length human HAAH cDNA was cloned into the pcDNA3 expression vector as well as a plasmid that encodes v-src which was used as a positive control for transformation activity. Standard methods were used for transfection of NIH 3T3 cells, control for transfection efficiency, assays of HAAH enzymatic activity, transformation by analysis of foci formation, anchorage-independent cell growth assays and analysis of tumorigenicity in nude mice. The data indicate that HAAH overexpression is associated with generation of a malignant phenotype.

Table 4: Overexpression of enzymatically active HAAH indicates malignancy

cDNA	# of foci $\pm$ S.D. <sup>b</sup>	NIH 3T3 clone	# of colonies <sup>e</sup>
pcDNA3 (mock)	6.0 $\pm$ 3.3	pcDNA (mock)	0.4 $\pm$ 0.5
murine HAAH	14.0 $\pm$ 2.9	clone 18 <sup>d</sup>	6.2 $\pm$ 2.9
mutant murine HAAH <sup>a</sup>	1.6 $\pm$ 1.0	clone 16 <sup>e</sup>	4.7 $\pm$ 6.5
human HAAH	32.0 $\pm$ 5.4		
v-scr	98.0 $\pm$ 7.1		

a. enzymatically inactive HAAH

b. P<0.01 compared to mock and mutant murine HAAH

c. P<0.001 compared to mock

d. Clone 18 is a stable cloned NIH 3T3 cell line that overexpression human HAAH by approximately two fold.

e. Clone 16 is a stable cloned NIH 3T3 cell line that overexpresses human HAAH by about 50%.

These data indicate that overexpression of HAAH is associated with formation of transformed foci. Enzymatic activity is required for cellular transformation to occur. Cloned NIH 3T3 cell lines with increased human HAAH gene expression grew as solid tumors in nude mice. HAAH is a downstream effector gene of the IRS-1 signal transduction pathway.

#### Example 5: Inhibition of HAAH gene expression

The FOCUS HCC cell line from which the human HAAH gene was initially cloned has a level of HAAH expression that is approximately 3-4 fold higher than that found in normal liver. To make an HAAH antisense construct, the full length human HAAH cDNA was inserted in the opposite orientation into a retroviral vector containing a G418 resistant gene, and antisense RNA was produced in the cells. Shorter HAAH antisense nucleic acids, e.g., those

corresponding to exon 1 of the HAAH gene are also used to inhibit HAAH expression.

FOCUS cells were infected with this vector and the level of HAAH was determined by Western blot analysis. A reduction in HAAH gene expression was observed. Growth rate and morphologic appearance of cells infected with a retrovirus containing a nonrelevant Green Fluorescent Protein (GFP) also inserted in the opposite orientation as a control (Fig. 8). Cells (harboring the HAAH antisense construct) exhibited a substantial change in morphology characterized by an increase in the cytoplasm to nuclear ratio as well as assuming cell shape changes that were reminiscent of normal adult hepatocytes in culture. Cells with reduced HAAH levels grew at a substantially slower rate than retroviral infected cells expressing antisense (GFP) (control) as shown in Fig. 8. A reduction in HAAH gene expression was associated with a more differentiated noncancerous "hepatocyte like" phenotype. Expression of HAAH antisense sequences are used to inhibit tumor growth rate. Reduction of HAAH cellular levels results in a phenotype characterized by reduced formation of transformed foci, low level or absent anchorage independent growth in soft agar, morphologic features of differentiated hepatocytes as determined by light and phase contrast microscopy, and no tumor formation (as tested by inoculating the cells into nude mice).

#### Example 6: Human IRS-1 mutants

Insulin/IGF-1 stimulated expression of HAAH in HCC cell lines. Dominant-negative IRS-1 cDNAs mutated in the plextrin and phosphotyrosine (PTB) domains, and Grb2, Syp and PI3K binding motifs located in the C-terminus of the molecule were constructed. Human IRS-1 mutant constructs were generated to evaluate how HAAH gene expression is

upregulated by activation of the IRS-1 growth factor signal transduction cascade. Specific mutations in the C terminus of the hIRS-1 molecule abolished the various domains which bind to SH2-effector proteins such as Grb2, Syp and PI3K.

5 The human IRS-1 protein contains the same Grb2 and Syp binding motifs of 897YVNI (underlined in Table 5, below and 1180YIDL (underlined in Table 5, below), respectively, as the rat IRS-1 protein. Mutants of hIRS-1 were constructed by substitution of a TAT codon (tyrosine) with a TTT codon  
10 (phenylalanine), in these motifs by use of oligonucleotide-directed mutagenesis using the following primers: (5'-GGGGGAATTTGTCAATA-3' (SEQ ID NO:8) and 5'-GAATTTGTTAATATTG-3' (SEQ ID NO:9), respectively). The cDNAs of hIRS-1 (wild-type) and mutants (tyrosine  
15 897-to-phenylalanine and tyrosine 1180-to-phenylalanine) were subcloned into the pBK-CMV expression vector and designated as hIRS-1-wt, 897F,  $\Delta$ -Grb2), 1180F, and  $\Delta$ Syp.

Table 5: Human IRS-1 amino acid sequence

	MASPPESDGF	SDVRKVGYL	KPKSMHKRFF	VLRAASEAGG	PARLEYEENE	KKWRHKSSAP	61
20	KRSIPLESCF	NINKRADSKN	KHLVALYTRD	EHFAIAADSE	AEQDSWYQAL	LQLHNRAGKH	121
	HDGAAALGAG	GGGGSCSGSS	GLGEAGEDLS	YGDVPPGPAF	KEVWQVILKP	KGLGQTKNLI	181
	GIYRLCLTSK	TISFVKLNSE	AAAVVLQLMN	IRRCHSENF	FFIEVGRSAV	TGPGFEWMQV	241
	DDSVVAQNMH	ETILEAMRAM	SDEFPRSKS	QSSSNCSNPI	SVPLRRHHLN	NPPPSQVGLT	301
	RRSRTESITA	TSPASMVGGK	PGSFRVRASS	DGEGTMSRPA	SVDGSPVSPS	TNRTHAHRHR	361
25	GSARLHPPLN	HSRSIPMPAS	RCSPTSATSPV	SLSSSSTSGH	GSTSDCLFPR	RSSASVSGSP	421
	SDGGFISSDE	YGSSPCDFRS	SFRSVTPDSL	GHTPPARGE	ELSNYICMGG	KGPSTLTAPN	481
	GHYILSRGGN	GHRCTPGTGL	GTSPALAGDE	AASAADLDNR	FRKRTHSAGT	SPTITHQKTP	541
	SQSSVASIEE	YTEMMPAYPP	GGGSGGRLPG	HRHSAFVPTR	SYPEEGLEMH	PLERRGGHHR	601
	PDSSTLHTDD	GYMPMSPGVA	PVPSGRKGS	DYMPMSPKSV	SAPQOIINPI	RRHPQRVDPN	661
30	GYMMSPSGG	CSPDIGGGS	SSSSSSNAV	SGTSYGLWT	NGVGGHSHV	LPHKPPVES	721
	SGGKLLPCTG	DYMNMSVPGD	SNTSSPDCY	YGPEDPQHKP	VLSYSLPRS	FKHTQRPGE	781
	EEGARHQHLR	LSTSSGRLLY	AATADSSSS	TSSDSLGGY	CGARLEPSLP	HPHHQVLQPH	841
	LPRKVDTAAG	TNSRLARPTR	LSLGDPKAST	LPRAREQQQQ	QQPLLHPPEP	KSPGEYVNI	901
	FGSDQSGYLS	GPVAFHSSPS	VRCPSQLQPA	PREEETGTEE	YMKMDLGPGR	RAAWQESTGV	961
35	EMGRLGPAPP	GAASICRPTR	AVPSSRGDYM	TMQMSCPRQS	YVDTSPAAPV	SYADMRTGIA	1021
	AAEVSLPRAT	MAAASSSSAA	SASPTGPQGA	AELAHSLL	GGPQGPQGM	AFTRVNLSPN	1081
	RNQSASFVIRA	DPQGCRRRHS	SETFSSTPSA	TRVGNTPVFG	AGAAVGGGGG	SSSSSESVKR	1141
	HSSASFVIRV	LRPGELGGAP	KEPAKLCGAA	GGLGNLNYI	DLDLVKDFKQ	CPQECTPEPQ	1201
	PPPPPPPHQP	LGSGESSTR	RSSEDLSAYA	SISFQKQPED	RQ (SEQ ID NO:5; GENBANK		
40	Accession No. JS0670; pleckstrin domain spans residues 11-113, inclusive; Phosphate-binding residues include 46,						

465, 551, 612, 632, 662, 732, 941, 989, or 1012 of SEQ ID NO:5)

Table 6: Human IRS-1 cDNA

	cggcggcgcg	gtcggagggg	gccggcgcg	agagccagac	gccgcgcgtt	gttttggttg	61
5	gggctctcgg	caactctccg	aggaggagga	ggaggaggga	ggaggggaga	agtaactgca	121
	gcggcagcgc	cctcccgagg	aacaggcgtc	ttccccgaac	ccttcccaaa	cctcccccat	181
	ccccctctcg	ccttgctccc	ttccccctct	cccagccgcc	tggagcgagg	ggcagggatg	241
	agtctgtccc	tccggccggg	ccccagctgc	agtggctgcc	cggtatcggt	tcgcattgga	301
	aagccacttt	ctccacccgc	cgagatgggc	cgggatgggg	ctgcagagga	cgcgcgccgc	361
10	ggcggcggca	gcagcagcag	cagcagcagc	agcaacagca	acagccgcag	cgcgcgcgtc	421
	tctgcgactg	agctgggtatt	tgggcggctg	gtggcggctg	ggacgggttg	gggggtggag	481
	gaggcgaaag	aggagggaga	accccggtga	acgttgggac	ttggcaaccc	gcctccccct	541
	gcccaggat	atttaatttg	cctcgggaat	cgtctgttcc	agaggggaac	tcaggaggga	601
	aggcgcgcg	gcgcgcgcgc	tcctggaggg	gcaccgcagg	gacccccgac	tgctgcctcc	661
15	ctgtgcccga	ctccagccgg	ggcgacgaga	gatgcattct	cgtctcttcc	tggtggcggc	721
	ggcgggtgag	aggagacttg	gctctcggag	gatcggggct	gccctcacc	cggacgcact	781
	gcctccccgc	cggcgtgaag	cgccccgaaa	ctccggctcg	gctctctcct	gggctcagca	841
	gctgcgtcct	ccttcagctg	ccccctcccc	gcgcgggggg	cggcgtggat	ttcagagtcg	901
	gggttttctg	tgcttccagc	cctgttttga	tgtgcccggc	cgcggcgagg	agcctccgcc	961
20	ccccaccgg	ttgtttttcg	gagcctccct	ctgctcagcg	ttggtggtgg	cgggtggcag	1021
	atggcgagcc	ctccggagag	cgatggcttc	tcggacgtgc	gcaagggtgg	ctacctgcgc	1081
	aaacccaaga	gcattgcaca	acgcttcttc	gtactgcgcg	cggccagcga	ggctgggggc	1141
	ccggcgcgcc	tcgagtacta	cgagaacgag	aagaagtggc	ggcacaagtc	gagcgcccc	1201
	aaacgctcga	ttccccctga	gagctgcttc	aacatcaaca	agcgggtgta	ctccaagaac	1261
25	aagcacctgg	tggctctcta	caccggggac	gagcactttg	ccatcgcggc	ggacagcgag	1321
	gccgagcaag	acagctggta	ccaggctctc	ctacagctgc	acaaccgtgc	taaggggcac	1381
	cacgaaggag	ctgcggccct	cggggcggga	ggtggtgggg	gcagctgcag	cggcagctcc	1441
	ggccttggtg	aggctgggga	ggacttgagc	tacggtgacg	tgcccccagg	accgcgattc	1501
	aaagaggtct	ggcaagtgat	cctgaagccc	aagggcctgg	gtcagacaaa	gaacctgatt	1561
30	ggtatctacc	gcctttgcct	gaccagcaag	accatcagct	tcgtgaagct	gaactcggag	1621
	gcagcggcgc	tgggtgtgca	tgctgatgaac	atcaggcgct	gtggccactc	ggaaaaactc	1681
	ttcttcatcg	agggtgggpc	ttctgcegtg	acggggcccc	gggagttctg	gatgcagggtg	1741
	gatgactctg	tgggtggccca	gaacatgcac	gagaccatcc	tggaggccat	gcggggccatg	1801
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    aaaaaaaa (SEQ ID NO:6; GENBANK Accession No. NM 005544)

```

The double mutation of tyrosine 897 and 1180 was  
 constructed by replacement of 3'-sequences coding 897F by  
 the same region of 1180F using restriction enzymes NheI and  
 EcoRI, and this construct was called 897F1180F or  $\Delta$ Grb2  $\Delta$ Syp.  
 The expression plasmids were under control of a CMV promoter  
 (hIRS-1-wt,  $\Delta$ Grb2,  $\Delta$ Syp,  $\Delta$ Grb2,  $\Delta$ Syp and pBK-CMV (mock) and  
 linearized at the 3'-end of poly A signal sequences by MluI  
 restriction enzymes followed by purification. A similar  
 approach was used to change the tyrosine residue to

phenyalanine at positions 613 and 942 to create the double  
PI3K mutant construct ( $\Delta$ PI3K). The hIRS-1 mutants have a  
FLAG epitope (DYKDDDDK (SEQ ID NO:6) + stop codon) added to  
the C-terminus by PCR. This strategy allows to distinguish  
5 the mutant protein from "wild type" hIRS-1 in stable  
transfected cell lines. The mutants are used to define the  
link between the IRS signal transduction pathway and  
activation of HAAH as a downstream effector gene and  
identify compounds to inhibit transduction along the pathway  
10 to inhibit growth of tumors characterized by HAAH  
overexpression. Antibodies or other compounds which bind to  
phosphorylation sites or inhibit phosphorylation at those  
sites are used to inhibit signal transduction and thus  
proleferation of HAA-overexpressing tumors.

15 Other embodiments are within the following claims.  
What is claimed is:

1           1. A method for diagnosing a malignant neoplasm in a  
2 mammal, comprising contacting a bodily fluid from said  
3 mammal with an antibody which binds to an human aspartyl  
4 (asparaginy) beta-hydroxylase (HAAH) polypeptide under  
5 conditions sufficient to form an antigen-antibody complex  
6 and detecting the antigen-antibody complex.

1           2. The method of claim 1, wherein said neoplasm is  
2 derived from endodermal tissue.

1           3. The method of claim 1, wherein said neoplasm is  
2 selected from the group consisting of colon cancer, breast  
3 cancer, pancreatic cancer, liver cancer, and cancer of the  
4 bile ducts.

1           4. The method of claim 1, wherein said neoplasm is  
2 a cancer of the central nervous system (CNS).

1           5. The method of claim 1, wherein said bodily fluid  
2 is selected from the group consisting of a CNS-derived  
3 bodily fluid, blood, serum, urine, saliva, sputum, lung  
4 effusion, and ascites fluid.

1           6. The method of claim 1, wherein said antibody is a  
2 monoclonal antibody.

1           7. The method of claim 6, wherein said monoclonal  
2 antibody is FB50.

1           8. The method of claim 6, wherein said monoclonal  
2 antibody is selected from the group consisting of 5C7, 5E9,  
3 19B, 48A, 74A, 78A, 86A.

1           9. A method for prognosis of a malignant neoplasm  
2 of a mammal, comprising

3                 (a) contacting a bodily fluid from said mammal  
4 with an antibody which binds to an HAAH polypeptide under  
5 conditions sufficient to form an antigen-antibody complex  
6 and detecting the antigen-antibody complex;

7                 (b) quantitating the amount of complex to  
8 determine the level of HAAH in said fluid; and

9                 (c) comparing the level of HAAH in said fluid  
10 with a normal control level of HAAH, wherein increasing  
11 levels of HAAH over time indicates an adverse prognosis.

1           10. A method of inhibiting tumor growth in a mammal  
2 comprising administering to said mammal a compound which  
3 inhibits expression of HAAH.

1           11. The method of claim 10, wherein said compound is  
2 a HAAH antisense nucleic acid.

1           12. The method of claim 10, wherein said compound  
2 is a ribozyme.

1           13. The method of claim 10, wherein said tumor is  
2 derived from endodermal tissue.

1           14. The method of claim 10, wherein said tumor is  
2 selected from the group consisting of colon cancer, breast  
3 cancer, pancreatic cancer, liver cancer, and cancer of the  
4 bile ducts.

1           15. The method of claim 10, wherein said tumor is a  
2 CNS tumor.

1           16. A method of inhibiting tumor growth in a mammal  
2 comprising administering to said mammal a compound which  
3 inhibits an enzymatic activity of HAAH.

1           17. The method of claim 16, wherein said enzymatic  
2 activity is hydroxylase activity.

1           18. The method of claim 16, wherein said compound  
2 is a dominant negative mutant of HAAH.

1           19. The method of claim 18, wherein said dominant  
2 negative mutant HAAH comprises a mutation in a catalytic  
3 domain of HAAH.

1           20. The method of claim 16, wherein said compound  
2 is an HAAH-specific intrabody.

1           21. The method of claim 16, wherein said compound  
2 is L-mimosine.

1           22. The method of claim 16, wherein said compound  
2 is a hydroxypyridone.

1           23. A method of inhibiting tumor growth in a mammal  
2 comprising administering to said mammal a compound which  
3 inhibits signal transduction through the IRS signal  
4 transduction pathway.

1           24. The method of claim 23, wherein said compound  
2 inhibits IRS phosphorylation.

1           25. The method of claim 23, wherein said compound  
2 inhibits binding of Fos or Jun to an HAAH promoter sequence.

1           26. A method of inhibiting tumor growth in a mammal  
2 comprising administering to said mammal a compound which  
3 inhibits HAAH hydroxylation of a NOTCH polypeptide.

1           27. The method of claim 26, wherein said compound  
2 inhibits hydroxylation of an EGF-like repeat sequence in a  
3 NOTCH polypeptide.

1           28. A method of killing a tumor cell comprising  
2 contacting said tumor cell with cytotoxic agent linked to an  
3 HAAH-specific antibody.

1           29. A monoclonal antibody that binds to an epitope  
2 of HAAH.

1           30. The antibody of claim 29, wherein said epitope  
2 is within a catalytic site of HAAH.

1           31. The antibody of claim 29, wherein said  
2 monoclonal antibody is selected from the group consisting of  
3 5C7, 5E9, 19B, 48A, 74A, 78A, 86A.

1           32. The antibody of claim 29, wherein said  
2 monoclonal antibody is selected from the group consisting of  
3 HA238A, HA221, HA239, HA241, HA329, or HA355.

1           33. A composition comprising a monoclonal antibody  
2 that binds to an epitope of HAAH linked to a cytotoxic  
3 agent, wherein said composition preferentially kills tumor  
4 cells compared to non-tumor cells.

1           34. A kit for diagnosis of a tumor in a mammal,  
2 comprising the antibody of claim 29.



Abstract of the Disclosure

The invention features a method for diagnosing a malignant neoplasm in a mammal by contacting a bodily fluid from the mammal with an antibody which binds to an human aspartyl (asparaginy) beta-hydroxylase (HAAH) polypeptide and methods of treating malignant neoplasms by inhibiting HAAH.

402403.B11

**Fig. 1**

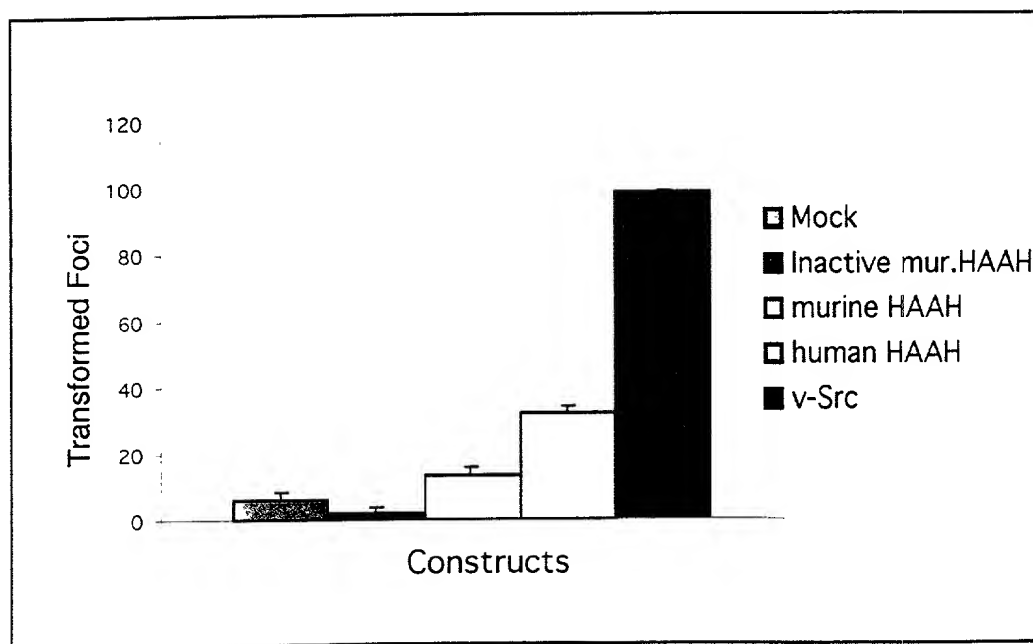
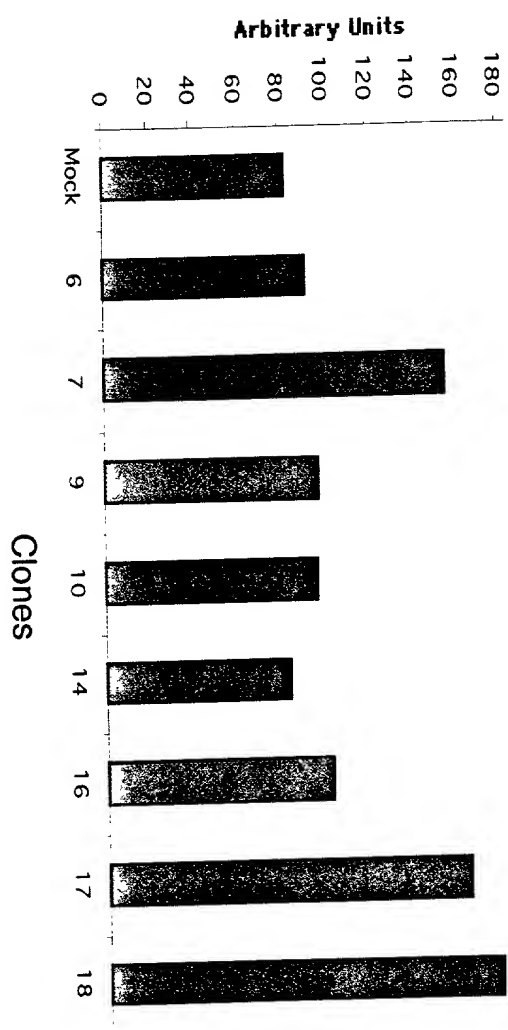


Fig. 2



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Fig. 3a

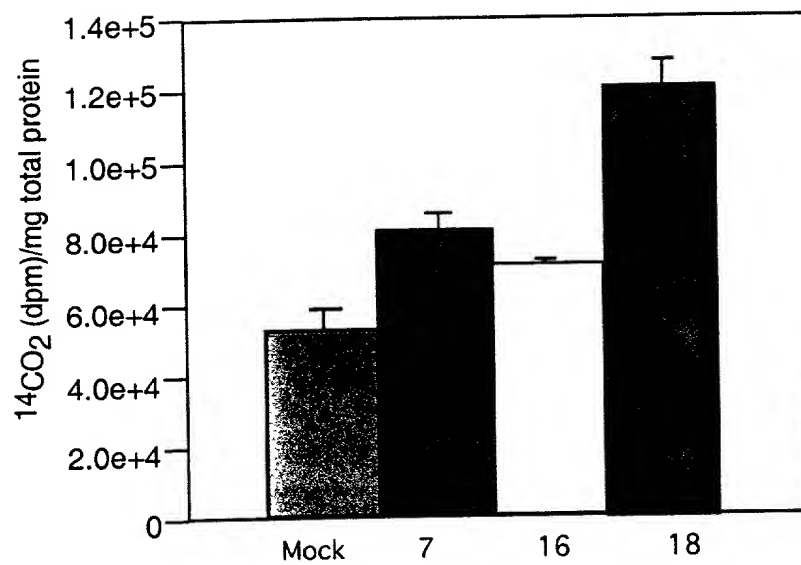
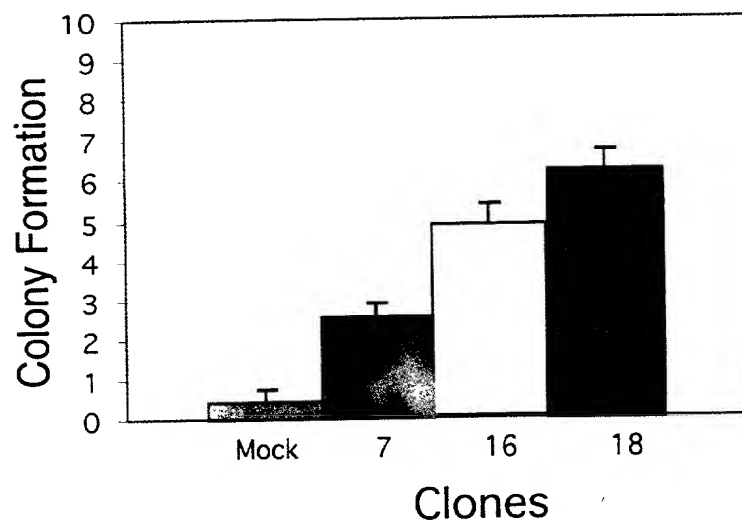


Fig. 3b



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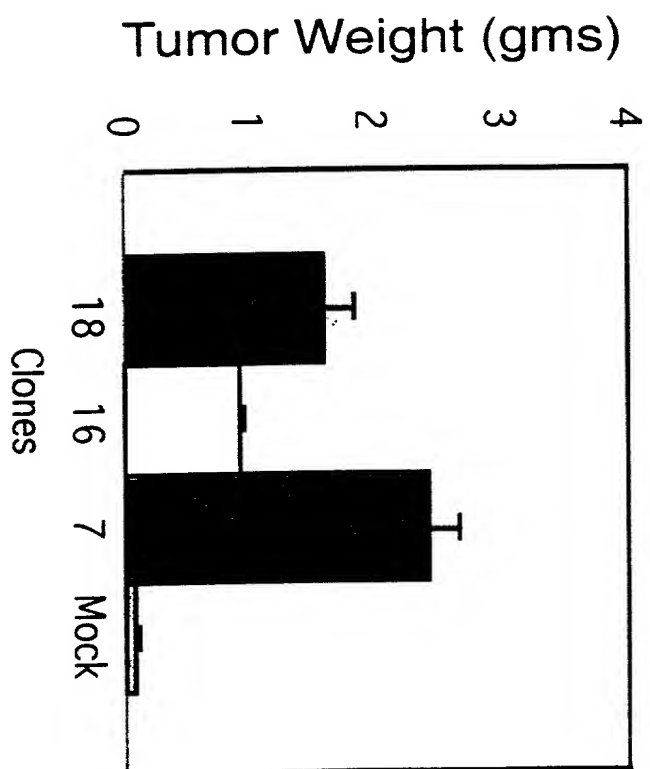


Fig. 5a

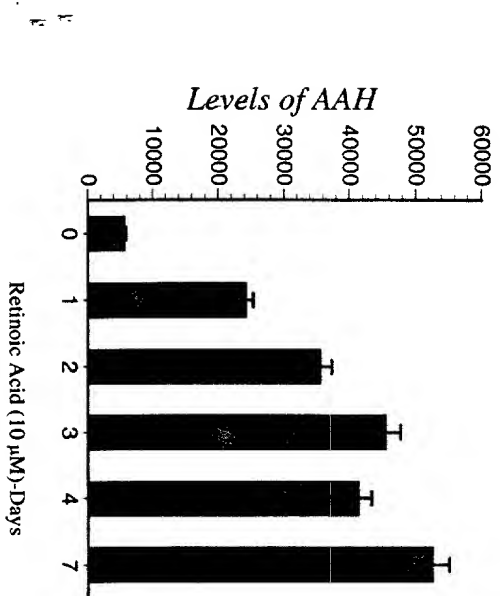


Fig. 5b

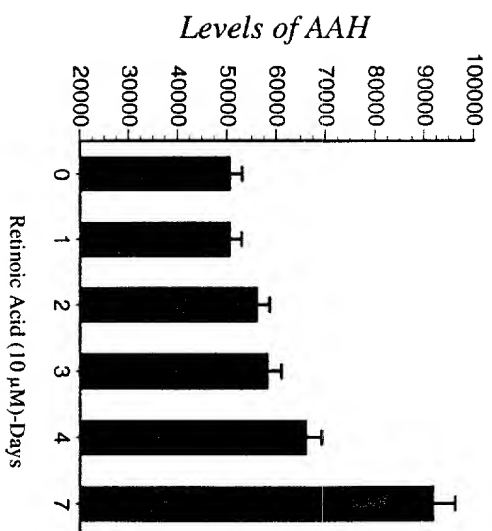


Fig. 5c

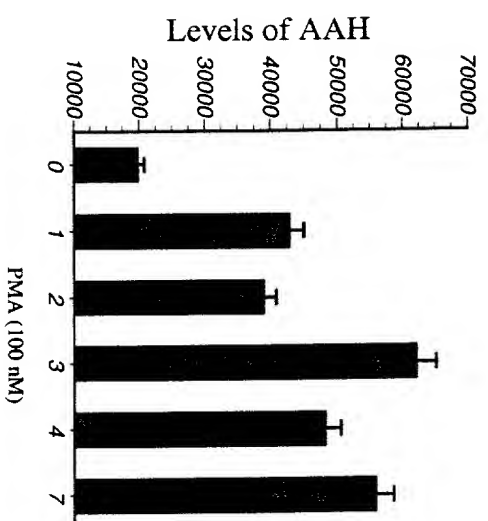
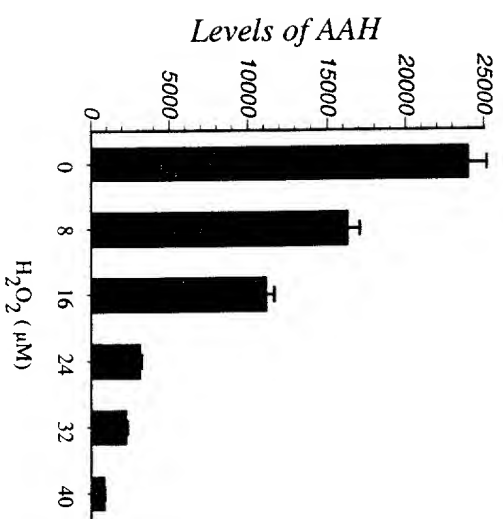
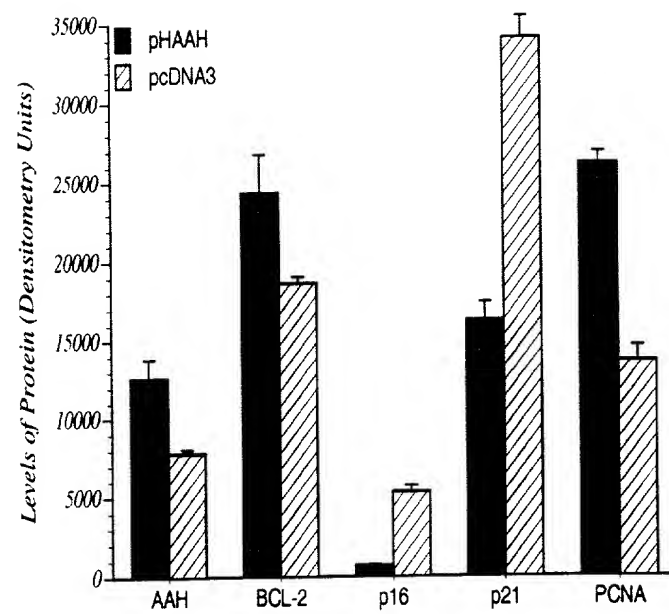


Fig. 5d



**Fig. 6**



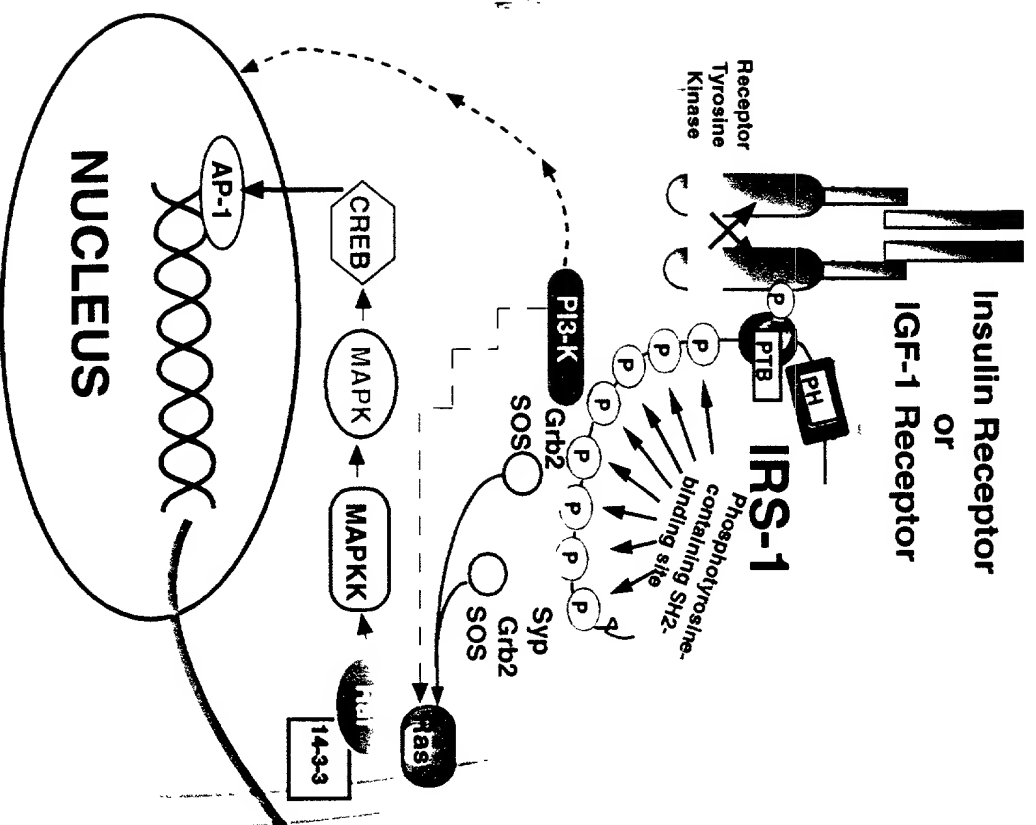


Fig. 7

1. Cell proliferation
2. Up-regulation of growth genes
3. Resistance to apoptosis
4. Increased Invasive Properties
5. Secondary Genetic Events

**Fig. 8**

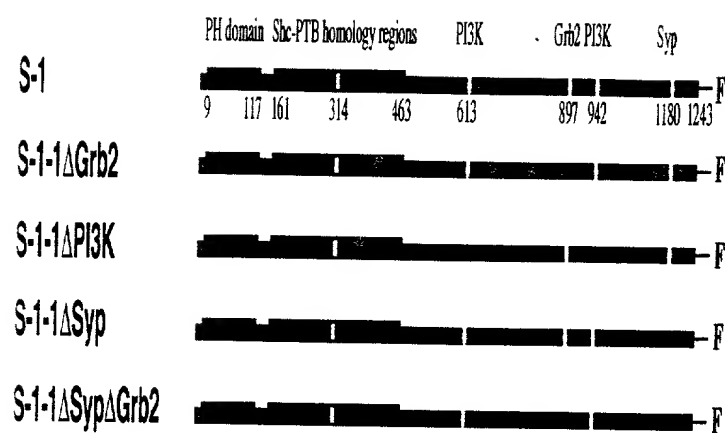
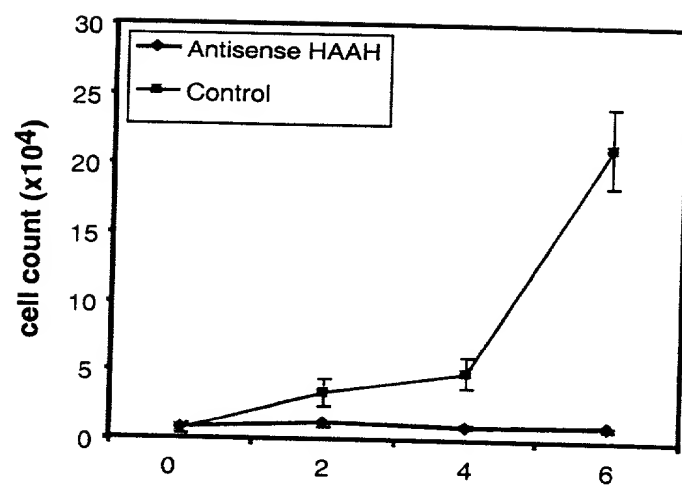


Fig. 9



## COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled DIAGNOSIS AND TREATMENT OF MALIGNANT NEOPLASMS, the specification of which:

☒ is attached hereto.  
☐ was filed on \_\_\_\_\_, as Application Serial No. \_\_\_\_\_ and was amended on \_\_\_\_\_.  
☐ was described and claimed in PCT International Application No. \_\_\_\_\_ filed on \_\_\_\_\_ and as amended under PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

U.S. Serial No.	Filing Date	Status

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

04930-032001

## Combined Declaration and Power of Attorney

Page 2 of 2 Pages

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